University of Alberta

Control Mechanisms of Mast Cell Derived Nitric Oxide

By

Mark Gilchrist

A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

In

Experimental Medicine

Department of Medicine

Edmonton, Alberta

Spring 2004

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 0-612-96270-9
Our file Notre référence
ISBN: 0-612-96270-9

The author has granted a non-exclusive license allowing the Library and Archives Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou aturement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

Abstract

Mast cells (MC) are specialized secretory cells found in diverse tissue environments often at host/environment interfaces. MC produce a broad repertoire of granule-associated and lipid-derived mediators and numerous cytokines. This stock of mediators implicates MC in multiple homeostatic responses including wound healing, angiogenesis and epithelial integrity. However, explosive activation following antigen binding of specific IgE molecules in inflammatory states such as asthma is the public face of this cell. Controlling MC activation is an important strategy in treating inflammation.

Previous studies show that nitric oxide (NO) can moderate MC responsiveness. We hypothesized that MC produce NO, which regulates MC functions. We investigated NO formation and expression of nitric oxide synthase (NOS) in MC populations. Rodent MC are a constitutive source of endothelial (eNOS) and neuronal (nNOS) mRNA while human MC express both mRNA and protein. eNOS protein localized to cytoplasmic and nuclear regions of human and rat MC, while nNOS was exclusively cytoplasmic. These proteins were active, as NOS activity was measured using a radioactive assay for the by-product, citrulline. Further analysis of NO formation with the fluorescent probe diaminofluorescein (DAF) showed constitutive NO formation localized in both cytoplasmic and nuclear regions <5min following activation in rat and human MC. This was associated with inhibition of degranulation and leukotriene release. We have shown previously that interferon-γ (IFN-γ) inhibits MC adhesion in a NO-dependent manner. Treatment of rat MC with IFN-γ for 18 h

increased inducible (iNOS) expression in the cytoplasm, with NO detected by DAF fluorescence in cytoplasmic and Golgi regions, and associated inhibition of granule-mediator release.

Concordant with increased iNOS was an increase in GTP-cyclohydrolase I (CHI) expression and activity, which enhanced the availability of the NOS cofactor, tetrahydrobiopterin (BH_4). Inhibition of this pathway decreased NO production and made MC hyperresponsive to stimuli. In human MC IFN-γ (18 h) increased eNOS expression and activity that depressed chemokine expression and release. These results highlight the inhibitory actions of NO on MC degranulation, leukotriene formation and cytokine/chemokine release. Thus, further understanding of the NO formation in MC populations may reveal unique pathways that can be pharmacologically manipulated to control MC-mediated inflammatory states.

Dedication

Brought to you by the letter "E"

"I'm gasoline……I'm burning clean…."

Michael Stipe

Preface

This thesis has been written in paper format according to the guidelines of the University of Alberta. Each chapter stands alone as a separate document and is written in the style of the Journal of Experimental Medicine. With no exception, the experimental data in this thesis was generated by Mark Gilchrist.

Acknowledgements

This degree has been a long one in developing, if not in the actually completion. Life is never linear in my experience and I often look on at the "curves and lines" of my Grand Designs, and reconfirm the non-conformity of it !

I begin by acknowledging my family for their support and understanding as I embarked and progressed over this road. Though you were never sure of all the details, you knew what I was doing was important to me, and that was all the information you required. Thank you Mom, Dad, Kay and Brendon for everything.

I am also indebted to my Supervisor, Dr. Dean Befus, for offering the opportunity to survey this parabola, and for the free-reign he extended me to explore the possibilities and too take some bold chances. I also would like to thank my Committee members, Dr. Marek Radomski and Dr. Irvin Mayers for their cooperation, input and critical analysis.

Due to the paths I have chosen, I have been in the unique position over the last eight years of being able to draw on, and contribute too, an extended lab-corporate of experiential and historical knowledge. It was the foundation of the avenues I approached (and some I avoided !), and offered a strong core to build this thesis on. I wish to thank Dr. Fiona Wills for discussions, insight, and the power of strong expectations and goals, Dr. Grant Stenton, for always being willing to lend a hand, Lynelle Watt, who deserves a degree for making all my arrangements for me (!) and Rene Dery and Nadir Hirji for help, hoops, and a long list of "CSI" stories.

Ones' Grad School odyssey is never complete without fellow students to share the hills and valleys. I thank Cory Ebeling, for late night rant sessions and the "Club PRG", and Scott McCauley, for adding new life to an old lab bay. In particular I wish to thank Mike "Ugly" Logan, for keeping me sane, keeping me in stitches, and generally making Grad School more than habitable ! I got now, you got next.

That's a wrap !!

Table of Contents

Chapter 1	General Introduction	1
I.	Mast cells	2
	A. Mast cell characteristics	2
	1. Morphology, ontogenesis and localization	2
	2. Heterogeneity	3
	3. Mast cell mediators	5
	a) Granule-derived mediators.	5
	b) Lipid-derived mediators	7
	c) Cytokines/chemokines	7
	B. Mast cell activation.	7
	1. IgE-dependent activation and signaling	8
	2. IgE-independent activation	9
	C. Mast cells in homeostasis	11
	D. Mast cells in disease	12
	E. Regulators of mast cell responsiveness	13
II.	Nitric oxide	14
	A. General properties	14
	B. Nitric oxide chemistry	15
	C. Nitric oxide synthase	17
	1. General	17
	2. Structure and function	17
	3. Expression and post-translational regulation	21
	a) Transcriptional regulation	21
	b) Cofactor regulation	23
	i) Arginine	23
	ii) Tetrahydrobiopterin (BH_4)	24
	c) Localization and protein/protein interactions	25
	i) Caveolin	25
	ii) Heat shock protein 90	25
	iii)Other nitric oxide synthase interacting proteins	26
	d) Phosphorylation	27
	D. Nitric oxide and mast cells	28
III.	Central hypothesis and aims	29
IV.	References for Chapter 1	31
Chapter 2.	Nitric Oxide Synthase and Nitric Oxide Production in In-Vivo Derived Mast Cells	52

I.	Introduction	53
II.	Materials and Methods	54
III.	Results 1. Analysis of nitric oxide synthase expression in unstimulated peritoneal mast cells 2. Analysis of inducible nitric oxide synthase messenger RNA expression by reverse-transcriptase polymerase chain reaction 3. Inducible nitric oxide synthase messenger RNA localization by in situ reverse-transcriptase polymerase chain reaction 4. Inducible nitric oxide synthase immunohistochemistry in peritoneal mast cells 5. Nitric oxide production in peritoneal mast cells 6. Nitric oxide synthase activity in peritoneal mast cells	59
IV.	Discussion	68
V.	References for Chapter 2	73
Chapter 3.	Tetrahydrobiopterin: Critical Factor in the Production and Role of Nitric Oxide in Mast Cells.	77
I.	Introduction	78
II.	Materials and Methods	79
III.	Results 1. Sustained (>18 h) intracellular production of nitric oxide in interferon-γ -treated peritoneal mast cells 2. Real-time confocal analysis of IgE-mediated degranulation and nitric oxide production 3. Ca^{2+} flux and nitric oxide production in peritoneal mast cells 4. GTP-cyclohydrolase I/GTP-cyclohydrolase I feedback regulatory protein messenger RNA expression in peritoneal mast cells 5. Differential regulation of GTP-cyclohydrolase I and GTP-cyclohydrolase I feedback regulatory protein messenger RNA 6. GTP-cyclohydrolase I protein expression	83

7. Immunofluorescence localization of GTP-cyclohydrolase I in peritoneal mast cells
8. GTP-cyclohydrolase I enzymatic activity
9. Control of peritoneal mast cell nitric oxide (>18 h) production by tetrahydrobiopterin
10. Effects of tetrahydrobiopterin modulation (>18 h) on mast cell degranulation
11. Effects of tetrahydrobiopterin inhibition on short-term (<5 min) nitric oxide production/mast cell degranulation

IV. Discussion 94

V. References for Chapter 3 100

Chapter 4. Expression, Localization and Regulation of Nitric Oxide Synthase (NOS) in Human Mast Cells: Effects on Leukotriene Production. 105

I. Introduction 106

II. Materials and Methods 106

III. Results 111

1. Nitric oxide synthase mRNA expression by human mast cells
2. Confocal microscopic analysis of nitric oxide synthase localization in human mast cells
3. Nitric oxide synthase protein expression in human mast cells
4. Nitric oxide synthase activity and nitric oxide formation in human mast cells
5. Activation and co-distribution of endothelial nitric oxide synthase and 5-lipoxygenase
6. 5-lipoxygenase and endothelial nitric oxide synthase colocalization in human mast cells
7. Effects of modulating nitric oxide formation on leukotriene production in human mast cells

IV. Discussion 121

V. References for Chapter 4 128

Chapter 5. Interferon-γ Regulates Chemokine Expression and Release in Human Mast Cells: Role of Nitric Oxide 134

I.	Introduction	135
II.	Materials and Methods	136
III.	Results **1.** Identification of interferon-γ regulated gene expression in human mast cell-1 by cDNA array **2.** Confirmation of interferon-γ regulation of nitric oxide synthase messenger RNA expression **3.** Nitric oxide synthase protein expression in interferon-γ treated human mast cell-1 **4.** Nitric oxide synthase activity and nitric oxide formation in human mast cell-1 **5.** Regulation of monocyte chemoattractant protein-1 and I-309 messenger RNA expression by nitric oxide 6. Regulation of monocyte chemoattractant protein -1 and I-309 release by nitric oxide	138
IV.	Discussion	146
V.	References for Chapter 5	151
Chapter 6.	Interferon-γ Regulates Protein Inhibitor of Nitric Oxide Synthase in Human Mast Cells	156
I.	Introduction	157
II.	Materials and Methods	158
III.	Results 1. cDNA array analysis of interferon-γ regulated gene expression in human mast cell-1 2. Confirmation of interferon-γ regulation of messenger RNA expression 3. Western blot analysis of protein inhibitor of nitric oxide synthase expression in human mast cell-1 4. Confocal analysis of protein inhibitor of nitric oxide synthase localization in human mast cell-1	160
IV.	Discussion	165
V.	References for Chapter 6	169

Chapter 7.	General Discussion and Conclusions	173
I.	Summary of data	174
II.	Nitric oxide synthase expression and heterogeneity in mast cells	174
III.	Defining nitric oxide production in mast cells	179
IV.	Compartmentalization and roles of nitric oxide synthase and nitric oxide in mast cells	181
	A. Sustained nitric oxide: Localization and targets	181
	B. Immediate nitric oxide: Localization and targets	182
V.	Is short-term nitric oxide a generator of functional and phenotypic diversity in mast cells ?	186
VI.	Does tetrahydrobiopterin production determine reactive species production from mast cell nitric oxide synthase ?	189
VII.	Conclusions	193
VII.	References for Chapter 7	195

List of Tables

Chapter 2

Table 2.I	Primer sets used for reverse-transcriptase polymerase chain reaction experiments	57
Table 2.II	Immunohistochemical analysis of inducible nitric oxide synthase expression in peritoneal mast cells	66

Chapter 5

Table 5.I	Genes significantly regulated by interferon-γ in HMC-1	140

Chapter 7

Table 7.I	Nitric oxide synthase expression in mast cell populations	175

List of Figures

Chapter 1

Figure 1.1	Mast cell morphology and mediators.	6
Figure 1.2	IgE signaling pathways.	10
Figure 1.3	Nitric oxide: spectrum of interactions.	16
Figure 1.4	Monomeric nitric oxide synthase structure.	18
Figure 1.5	Nitric oxide synthase enzymology.	20
Figure 1.6	Regulatory pathways of cellular nitric oxide production.	22

Chapter 2

Figure 2.1	Expression of nitric oxide synthase messenger RNA in unstimulated peritoneal mast cells.	60
Figure 2.2	Measurement of messenger RNA levels for inducible nitric oxide synthase and β-actin in peritoneal mast cells.	62
Figure 2.3	*In-situ* reverse-transcriptase polymerase chain reaction localization of inducible nitric oxide synthase messenger RNA in peritoneal mast cells.	64
Figure 2.4	Immunohistochemical identification of inducible nitric oxide synthase protein in peritoneal mast cells.	65
Figure 2.5	The measurement of nitric oxide production from peritoneal mast cells by the Griess assay.	67
Figure 2.6	Citrulline assay for nitric oxide synthase activity in peritoneal mast cells.	69

Chapter 3

Figure 3.1	Fluorescence detection and analysis of nitric oxide and calcium in peritoneal mast cells.	85
Figure 3.2	Semiquantitative reverse-transcriptase polymerase chain reaction analysis of GTP-cyclohydrolase I and GTP-cyclohydrolase I feedback regulatory protein messenger RNA expression.	88

Figure 3.3	Western blot and confocal analysis of GTP-cyclohydrolase I protein in peritoneal mast cells.	90
Figure 3.4	GTP-cyclohydrolase I enzymatic activity in peritoneal mast cells.	92
Figure 3.5	The effect of tetrahydrobiopterin modulation on peritoneal mast Cell nitric oxide production.	93
Figure 3.6	Effects of tetrahydrobiopterin availability on β-hexosaminidase release from peritoneal mast cells.	95
Figure 3.7	Real-time confocal analysis of the effects of tetrahydrobiopterin inhibition on mast cell nitric oxide production and degranulation.	96
Chapter 4		
Figure 4.1	Expression of nitric oxide synthase isoforms in human mast cell populations.	112
Figure 4.2	Localization of nitric oxide synthase in human mast cell-1 and LAD2 cells.	114
Figure 4.3	Immunoblot analysis of nitric oxide synthase protein expression in subcellular fractions from human mast cell-1.	115
Figure 4.4	Nitric oxide synthase activity and nitric oxide localization in human mast cell.	117
Figure 4.5A	Translocation of endothelial nitric oxide synthase and 5-lipoxygenase in human mast cell-1.	119
Figure 4.5B	Colocalization of endothelial nitric oxide synthase and 5-lipoxygenase in human mast cell-1.	120
Figure 4.6	Effect of nitric oxide synthase inhibition and exogenous nitric oxide on cysteinyl leukotriene production in human mast cells.	122
Chapter 5		
Figure 5.1	Interferon-γ regulation of nitric oxide synthase messenger RNA expression in human mast cell-1.	141
Figure 5.2	Interferon-γ regulation of nitric oxide synthase protein expression in human mast cell-1.	142

Figure 5.3	Nitric oxide synthase activity in human mast cell-1.	144
Figure 5.4	Monocyte chemoattractant protein-1 /I-309 messenger RNA regulation by interferon-γ and nitric oxide.	145
Figure 5.5	Effect of nitric oxide synthase inhibition and exogenous nitric oxide on monocyte chemoattractant protein-1 and I-309 release in human mast cell-1.	147
Chapter 6		
Figure 6.1	cDNA array results for interferon-γ-treated human mast cell-1.	162
Figure 6.2	Verification of cDNA array results by reverse-transcriptase polymerase chain reaction .	163
Figure 6.3	Protein inhibitor of nitric oxide synthase expression and localization in human mast cell-1.	164
Figure 6.4	Colocalization of protein inhibitor of nitric oxide synthase and nitric oxide synthase in human mast cell-1.	166
Chapter 7		
Figure 7.1	Model of nitric oxide synthase expression in mast cell heterogeneity.	178
Figure 7.2	Long-term (hours) effects of upregulated nitric oxide synthase activity on mast cell responses.	183
Figure 7.3	Short-term (<30 min) effects of localized nitric oxide formation.	185
Figure 7.4	Generation of diversity by short-term nitric oxide production.	188
Figure 7.5	Proposed role for nitric oxide synthase/tetrahydrobiopterin in mast cell activation.	192

List of Abbreviations

3-NT	3-nitrotyrosine
48/80	compound 48/80
5-LO	5 lipoxygenase
A23187	calcium ionophore
AA	arachidonic acid
Ag	antigen
AL	argininosuccinate lyase
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
AS	argininosuccinate synthase
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
BH_4	tetrahydrobiopterin
β-hex	β-hexosaminidase
BME	β-mercaptoethanol
BMMC	bone marrow-derived mast cells
Ca^{2+}	calcium
CaMKII	calmodulin kinase
CAT-2B	cationic amino acid transporter
cDNA	complementary deoxyribonucleic acid
CD34	cluster of differentiation 34
cGMP	cyclic 3', 5'-guanosine monophosphate
CHI	GTP cyclohydrolase I
c-kit	receptor for stem cell factor
c-kit ligand	stem cell factor
cNOS	constitutive NOS
COX	cyclooxygenase
CTMC	connective tissue mast cell
DAF	diaminofluorescein
DAG	diacylglycerol
DAHP	2,4-diamino-6-hydroxypyrimidine
DEPC	diethyl pyrocarbonate
DLC	dynein light chain
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
ERK	extracellular receptor activated kinase
ER	endoplasmic reticulum

F	fluorescence obtained after activation
ΔF	change in fluorescence from baseline
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
FcεRI	high affinity Fc-epsilon receptor-I
FcγRIIB	Fc-gamma receptor II B
FMN	flavin mononucleotide
GEF	guanine exchange factor
GFRP	GTP-CHI feedback regulatory protein
GSNO	s-nitrosoglutathione
GTP	guanosine triphosphate
GTP-CHI	GTP cyclohydrolase I
HIMC	human intestinal mast cells
HMC-1	human mast cell line-1
HPLC	high performance liquid chromatography
HSMC	human skin mast cell
Hsp 90	Heat shock protein 90
HTB	HEPES Tyrode's buffer
I-309	CCL1 chemokine
IFN-γ	interferon-gamma
IgE	immunoglobulin E
IκB	inhibitory factor kappa-B
IL-1β	interleukin 1 beta
IL-3	interleukin-3
IL-4	interleukin-4
IL-5	interleukin-5
IL-10	interleukin-10
IMMC	intestinal mucosal mast cell
iNOS	inducible nitric oxide synthase
IP_3	inositol triphosphate
IRS	inhibitory receptor superfamily
ITAM	immunoreceptor tyrosine activation motif
ITIM	immunoreceptor tyrosine inhibition motif
JAK	Janus kinase
L-NAME	Nitro L-arginine methyl ester
L-NMMA	NG-monomethyl-L arginine
LPR	late-phase response
LPS	lipopolysaccharide
LTC_4	leukotriene C_4
MAFA	mast cell function-associated antigen

MAPK	mitogen-activated protein kinase
MC	mast cell
MC^C	mast cell chymase positive
MCP-1	monocyte chemoattractant protein 1
MC^T	mast cell tryptase positive
MC^{TC}	mast cell tryptase and chymase positive
MMC	mucosal MC
MMP	matrix metalloproteinases
N_2O_3	dinitrogen trioxide
NADPH	nicotinamide-adenine dinucleotide phosphate (reduced)
NAS	n-acetylserotonin
NMDA	N-methyl-D-aspartic acid
NO	nitric oxide
NOS	nitric oxide synthase
NOSIP	eNOS interacting protein
NOSTRIN	eNOS traffic inducer protein
nNOS	neuronal nitric oxide synthase
O_2^-	superoxide
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
$ONOO^-$	peroxynitrite
PAR-2	proteinase-activated receptor 2
PCR	polymerase chain reaction
PDZ	protein-protein binding domain
PGD_2	prostaglandin D_2
PGN	peptidoglycan
PIN	protein inhibitor of NOS
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLCγ	phospholipase C gamma
PMA	phorbol 12-myristate 13-acetate
PMC	peritoneal mast cell
RBL-2H3	rat basophilic leukemia cells
RCMC	rat cultured MC
RMCP-1	rat mast cell protease-1
RNS	reactive nitrogen species
RT	reverse transcriptase
SCF	stem cell factor
sGC	soluble guanylate cyclase
SNAP	S-nitroso-N-acetylpenicillamine
SNOG	S-nitrosoglutathione

TGF–β	transforming growth factor-beta
TLR	Toll-like receptor
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
WE	worm equivalent

Chapter 1

General Introduction

Introduction

The human immune response represents a highly integrated system that operates to combat the myriad of pathogenic and infectious agents present in our environment. Mammalian host defense has evolved into a complex, multi-cellular, multi-factorial system that provides both non-specific (innate) and highly adaptive responses to invading organisms (1).

Amongst the spectrum of cells involved in both innate and adaptive immunity, mast cells (MC) exist at a bifurcation point, contributing significantly to both classes of host defense (1). Indeed, there exists few disease states, or biological conditions that do not contain a MC component (2). However, even with the growing body of evidence about the significance of MC, much is still unknown as to their complete biological function despite a growing understanding of their activation and regulatory pathways (3).

I. Mast cells

A. Mast cell characteristics.

1. Morphology, ontogenesis and localization.

In the late 19th century, a young Paul Ehrlich noted a population of cells that stained strongly with his aniline dyes. To describe these cells he used the term "mastzellen" which means "well-fed cells" in the belief that the stained material had been phagocytosed (3). The metachromasia he noted, which is due to heparin, was the original defining characteristic of the MC.

Morphologically, MC size can be heterogeneous and vary in diameter from 10-20 μm. Although the cell may be round, oval, or spindle-shaped, mast cells characteristically have finger-like cytoplasmic projections (4). The purpose of these projections is unclear but may be to increase cellular surface area for greater adhesive or ligand-binding interactions. Ultrastructurally, MC contain the basic organelles needed for cellular metabolism including mitochondria, endoplasmic reticulum and also a relatively large nucleus which creates a low cytoplasmic to nuclear ratio consistent with cellular maturity (5). Notably, the cytoplasm is characteristically packed (>100/cell) with round, membrane-bound granules, the origin of Ehrlich's seminal description (Fig. 1.1).

MC are tissue-resident cells that arise from pluripotent $CD34^+$ bone marrow precursors and exit into the circulation in a committed though incompletely differentiated form (6). Conversely, basophils, which share many common features with MC, complete their hematopoetic maturation within the bone marrow. Human circulating MC precursors have a surface expression phenotype that is $CD34^+$, c-kit $(CD117)^+$, LY^-, $CD17^-$ and $CD14^-$. MC precursors home to target tissues and differentiate, granulate and mature in these distant tissue sites in response to distinct environmental factors (7). As all MC are positive for c-kit, the critical environmental factor in MC differentiation is stem cell factor (SCF or c-kit ligand). SCF induces MC proliferation and differentiation and is a key force in determining MC phenotype (8). However, studies using SCF mutant mice have shown that MC can still be found in the intestinal mucosa after prolonged *T. spiralis* infection. Such proliferation involves the cytokines IL-3 and IL-4 (in the absence of SCF) and indicates a T-cell dependency in sculpting MC maturation (9).

Due to these exclusive tissue environmental requirements for maturation, MC are found in all connective tissue, serosal and mucosal surfaces (10). As a result they are strategically located at neural, epidermal, mucosal, and vascular sites. MC can consistently be found in close proximity to blood vessels and nerve endings (11). Indeed, there are few microenvironments that are free of MC, though the vitreous humor of the eye and solid bone are two known examples (3).

2. Heterogeneity.

Owing to the unique effect that microenvironmental influences have on MC maturation it has long been known that, while MC in different sites share characteristics, they are heterogeneous. Indeed, Ehrlich's histochemical results gave the first clue that MC from different sites had unique staining properties, that was clarified by rodent studies showing staining that was variably sensitive to fixation (12).

Due to difficulties in obtaining pure populations of MC, the first viable system of heterogeneic nomenclature originated from localization and morphological criteria in rodents. Connective tissue MC (CTMC), as can be purified from skin and peritoneal cavity, and mucosal MC (MMC) derived from

intestinal lamina propria (13). These morphological differences were expanded to phenotypic and functional differences as studies progressed. These revealed that rat CTMC are larger, contain heparin as the granule proteoglycan, produce higher levels of histamine, express the protease RMCP-1 and respond to a variety of stimuli; while rat MMC are smaller, contain chondroitin sulfate as the granule proteoglycan, express the protease RMCP-II and are less responsive to various secretegoges (3). By nature this categorization is polarized and represents cells at extremes of the differentiation spectrum. Indeed rodent MC likely represent a continuum between CTMC and MMC.

Similar histochemical clues pointed to parallel heterogeneity in human MC, which also show variations in size, granule structure and quantities of mediators stored (13). Although similarities to rodent MC are noted, there are differences between MC heterogeneity in humans and in rodents. Central to defining distinct MC populations in human is the MC-specific granule protease content. As human MC only express one chymase, one subpopulation of human MC contain tryptase and chymase (MC^{TC}), while another contains only tryptase (MC^{T})(14). The distribution of MC subsets in human tissues is not as distinct as in rodents, though MC^{T} predominate in the lung (~93%), while MC^{TC} are found in skin (~99%) and intestinal submucosa (~80%)(15). Furthermore, several studies have identified a small subset of human MC that express only chymase (MC^{C}) (16). These results highlight the controversy that exists in defining human MC subsets, as the populations identified can vary with site of isolation, method of isolation or culture conditions (17,18). However, using localization analogy to rodent MC, MC^{T} are similar to MMC and MC^{TC} are similar to CTMC. As noted previously in rodents, microenvironmental influences can generate functional heterogeneity as well as a morphological heterogeneity in MC.

An understanding of MC heterogeneity is important, as physiological modulators and pharmacological agents may have variable outcomes on MC responses, ranging from complete inhibition to no effect, all as a result of MC phenotypic diversity.

3. Mast cell mediators.

The noted biological effects of MC activation result from release of a wide variety of potent mediators. This is particularly noticeable in inflammatory settings were MC-derived products released by classical degranulation/secretion have immediate effects on bronchomotor tone and mucous secretion (3,19). In addition MC can remodel plasma and nuclear membrane phospholipids to create lipid-derived inflammatory mediators. Finally, through *de novo* protein synthesis MC can produce cytokines that modulate cellular influx (1). Thus, similar to Cerberus the powerful three-headed dog of Hades, MC mediators can be categorized into three prongs: preformed granule-associated, lipid-derived and cytokines (Fig.1.1). Notably, MC have the capacity to differentially release these mediators depending on the stimulus (3,20).

a) Granule-derived. MC granules are membrane bound and have a proteoglycan core. As has been noted, significant proteoglycan heterogeneity exists in MC populations. In general terms heparin and chondroitin sulfate are the major MC proteoglycans (3). The unique chemical characteristics of proteoglycans (repeating serine/glycine residues with unbranched carbohydrate chains) possess a strong negative charge which allows for the packaging of granule mediators intracellularly (21).

The best-known preformed mediator is the amine, histamine, formed by the decarboxylation of histidine (3). Once produced histamine is tightly packaged with proteoglycans in the acidic environment of the granule. Upon MC activation and granule release, pH changes cause histamine liberation from the granule by cationic exchange (22) with rapid effects of histamine on many targets including smooth muscle and endothelium.

As previously noted, MC also express a variety of granule proteases. In rats these include RMCP-I (CTMC) and RMCP-II (MMC), which have chymase-like substrate specificity. The range of protease content in rodent MC is evolving into a complex picture with the cloning in mouse MC of multiple chymases (6) and tryptases (2) (23). In human MC, tryptase (MC^{T}) and chymase (MC^{TC}) are the

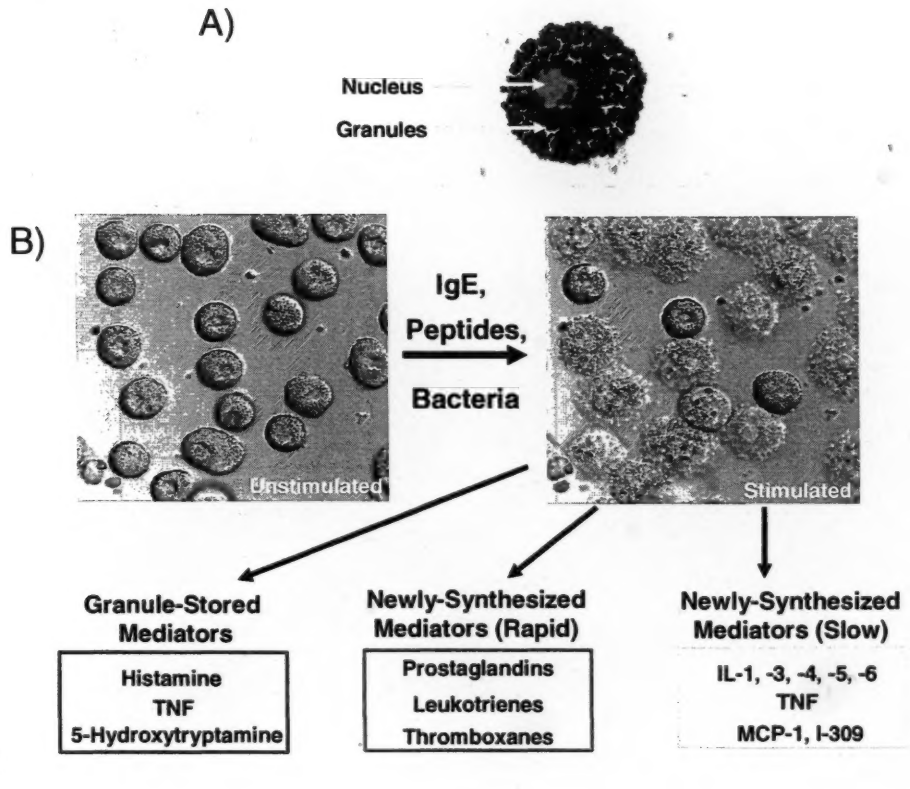


Fig. 1.1 Mast cell: morphology and mediators. A) Light microscope photograph of a Giemsa stained rat peritoneal mast cell showing large, off-center nucleus and dense staining cytoplasmic granules (800 X). B) Confocal image of rat peritoneal mast cells before and after activation. Mast cells can be activated though numerous pathways including crosslinking of IgE molecules bound to the high affinity IgE receptor, binding of cationic peptides or interaction with bacterial products. Mast cells can immediately release granule-stored mediators (degranulation shown in second confocal image) such as histamine and tumor necrosis factor (TNF), as well as rapidly (< 30 min) release de novo synthesized lipid mediators such as leukotrienes, and produce newly-synthesized mediators such as cytokines (Interleukin-; IL-) and chemokines (monocyte chemoattractant protein-1; MCP-1) with slower kinetics (> 1 h)(450 X).

predominant proteases, though human protease expression is likely more complex particularly at the tryptase locus and expression heterogeneity in tissue has been incompletely studied (3). These proteases are also granule-associated and it is believed they interact with heparin side chains (24). Proteases release has a multitude of effects including breakdown of basement membrane and matrix proteins as well as stimulating mucous secretion (25).

b) Lipid-derived mediators. MC can rapidly synthesize, *de-novo*, an array of potent inflammatory arachidonic acid (AA) metabolites. Upon MC activation, AA is liberated from plasma and nuclear membranes by the enzyme phospholipase A_2 (PLA_2)(26). Further sequential metabolism is undertaken by cycloxygenase (COX 1 and 2) to form prostaglandins and thromboxanes or lipoxygenase (LO) enzymes to generate leukotrienes (27,28). These mediators are potent bronchoconstrictors and chemotactic factors for multiple cell types. As with other MC mediators, there appears to be substantial heterogeneity in AA metabolites released by different MC populations. For example, rat CTMC preferentially produce PGD_2, whereas rat MMC produce LTB_4 and LTC_4 in addition to PGD_2, while human lung MC produce more LTC_4 and human skin MC more PGD_2(29). Again, microenvironmental factors, including cytokine exposure, are thought to contribute to this diversity (30).

c) Cytokines/chemokines. Finally, MC are also a source of "newly-synthesized" mediators including multiple cytokines (eg. IL-1, -3, -4, -5, IFN-γ, TNF) and chemokines (eg. MCP-1, I-309) (31,32). These molecules in general require new protein synthesis and thus are generally produced and released at later time points, although some are preformed and constitutively produced (eg. TNF)(Fig. 1.1)(33).

B. Mast cell activation.

MC can be activated by numerous secretagogues, that can induce mediator release though a variety of signaling cascades. The best-known inducer of MC secretory responses is though antigenic aggregation of IgE molecules bound to the high-affinity receptor for IgE (FcεRI) (34). However, addition pathways of activation are known and include a number of charged peptides in an IgE-

independent process known as peptidergic activation, as well as bacterial activation of MC through Toll-like receptors (TLR) (35,36).

Activation and degranulation of MC has several key features, including phosphorylation events, cytoskeletal changes, along with alterations in membrane phospholipid metabolism and changes in the level of intracellular calcium (Ca^{2+}) levels (37). Indeed, early studies showed that granule release was largely dependent on the presence of intracellular Ca^{2+}. Measurements of Ca^{2+} after MC activation show a biphasic response, with a small initial increase thought to arise from the release of Ca^{2+} from intracellular stores. Followed by a delayed, but substantial increase thought to be the result of Ca^{2+} influx across the plasma membrane (37).

1. IgE-dependent activation and signaling.

Cells of hematopoetic origin must express a variety of receptors that receive and transmit signals from the extracellular milieu. Among these receptor systems, antigen binding receptors are unique in that they posses the capacity to specifically bind to a diversity of ligands and this promotes the initiation of a specific immune response (38).

All MC populations express FcεRI. In rodents FcεRI is expressed as a heterotetramer consisting of a single IgE binding α-subunit, a β-subunit, and two disulfide-linked γ-subunits (39). The α-chain constitutes the major extracellular component of the receptor but lacks any known signal transducing domains (40). The β-subunit consists of 4 membrane spanning domains and a cytoplasmic tail capable of transducing cellular signals (40). The signaling competent γ-subunit is required for ligand induced signal transduction, and interacts with regions of the α-subunit (41).

Both the β and γ-subunits contain a specific sequence known as the immunoreceptor tyrosine-based activation motif (ITAM) that are important "phosphoacceptors", through which the receptor subunits ultimately interact with signaling proteins.

MC activated by FcεRI crosslinking show a very rapid (<15 sec) tyrosine phosphorlyation of many protein types (42), including the src related kinase, Lyn, and the tyrosine kinase, Syk. Lyn has been shown to phosphorylate β subunits of

FcεRI through ITAM motifs. Similar to other src family kinases, Lyn autophosphorylates itself, leading to further transmission to other downstream substrates (37). Substrates containing src-homology-2 (SH2) domains can now bind to the phosphorylated Lyn. Syk binds to the tyrosine phosphorylated β and γ ITAM's through its tandem SH2 domains (43). Recruitment of Syk occurs upstream of several important signal transduction pathways and is an important branch point in MC activation (Fig. 1.2) (39).

One of the best-characterized signaling cascade downstream of Syk involves the phosphorylation and activation of phospholipase C-γ (PLCγ) (37). PLCγ activation results in the hydrolysis of membrane phosphatidyl-inositol 4,5-biphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 initiates the release of Ca^{2+} from intracellular stores, while DAG activates numerous protein kinase C (PKC) isoforms (44). The increase in intracellular Ca^{2+} and PKC activation contribute directly to immediate MC mediator secretion.

Another exocytotic signaling pathway thought to arise from Syk activation is the Ras pathway. Ras activation requires the activation of the guanine exchange factor (GEF) Vav (45). Vav is known to form multimeric signaling complexes with many adaptor proteins including Shc, Grb2 and Sos (45). This complex interaction mediates the downstream activation of mitogen-activated protein kinase (MAPK) by Ras (39). Activation of MAPK is known to regulate phospholipase A_2 (PLA_2), and PLA2 activation promotes the production and release of AA metabolites (46). This pathway is a predominant contributor to the production of AA in MC (Fig. 1.2).

2. IgE-independent activation.

In addition to activation by FcεRI, MC are known to be activated by a family of polybasic molecules including compound 48/80 (48/80), mastoparan, and polymyxin B as well as by several peptides, including substance P and neutrophil defensin molecules (47-49). At present there is accumulating evidence that there are no specific receptors for these molecules (50).

These amphiphilic peptides share similar properties as triggers of MC degranulation. These include rapid kinetics (<10 sec), desensitization after neuraminidase pretreatment (removal of sialic acid), inhibition by pertussis toxin

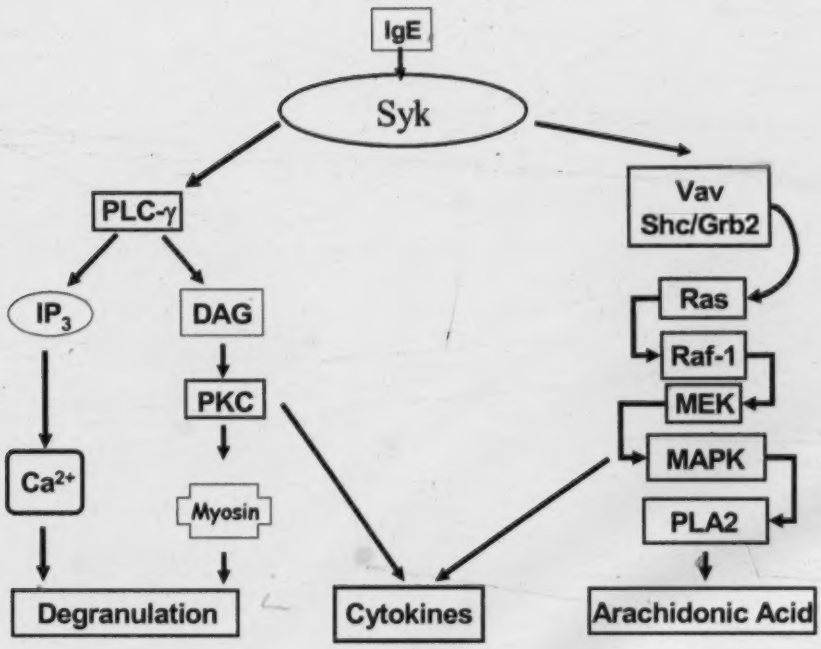


Fig. 1.2 Model of IgE-associated signaling pathways in mast cells. IgE-mediated signaling is centered on splenic tyrosine kinase (Syk) phosphorylation. Syk then activates multiple pathways including; phospholipase C-γ (PLC-γ), which liberates inositol-3-phosphate (IP_3) and diacylglycerol (DAG) causing calcium (Ca^{2+}) release and protein kinase C (PKC) activation respectively leading to cytoskeletal changes and degranulation; Ras, which activates the mitogen-activated protein kinase (MAPK) pathway leading to phospholipase A_2 (PLA_2) activation and arachidonic acid (AA)-derived mediator production. Several downstream targets of both PKC and MAPK are transcription factors involved in regulating cytokine expression.

pretreatment, and the transient stimulation of IP_3 formation (51). Direct G-protein activation is thought to be the mechanism involved, as treatment with pertussis toxin inhibits histamine secretion by MC treated with C48/80 (52).

One of the central roles played by IgE-dependent activation is the immune response necessary for the expulsion of parasites (1). In addition, studies using MC-deficient mice have shown that MC-derived TNF is critical in the innate defense against bacterial infections (53). Mammalian "Toll-like receptors" (TLR) have been shown to bind to conserved structural patterns of pathogenic bacteria (54). There are ten identified TLR and signaling via these receptors results in the production of an array of pro-inflammatory mediators (55). TLR2 and TLR4 have been extensively studied and respond to Gram-positive bacterial products (peptidoglycan) and Gram-negative enterobacterial lipopolysaccharide (LPS) respectively.

MC from rodents express TLR 2, 4, 6 and 8 but not TLR5 (36), while human MC TLR expression has been controversial with TLR 1,2,4 and 6 being variably identified (56,57). Rodent MC can be differentially activated by PGN and LPS to release a unique spectrum of mediators. In addition, PGN treatment of mouse MC caused granule–derived mediators to be released (β-hexosaminidase; β-hex) while LPS did not (20). Results from human MC studies have shown a similar trend, though evidence for TLR4 activation by LPS requires further confirmation. These studies show that MC can respond to bacterial products with distinct patterns of mediator release.

C. Mast cells in homeostasis.

MC activated through the above mechanisms play a critical role in immediate hypersensitivity inflammatory responses. This unfortunately draws attention away from the growing body of evidence of a role for MC in tissue homeostasis and repair. However, their ubiquitous distribution, plethora and differential release of potent mediators, and increased numbers in wounds, fractures, tumor sites and other inflammatory states support the concept that MC participate in multiple facets of tissue regulation (3).

Several lines of evidence point to MC involvement in wound healing and fibrosis. MC numbers diminish early at a wound site, followed by marked hyperplasia then slow return to normal numbers as healing progresses (58). In addition, MC secrete a variety of mediators essential for initiation of wound healing, including release of histamine that increases vascular permeability and causes collagen deposition (59); cytokines and chemokines that recruit other inflammatory cells to the site (32); proteases that induces fibroblast, endothelial and epithelial cell proliferation (59); and posses the ability to degrade and produce extracellular matrix proteins (3).

Another feature necessary for tissue homeostasis after injury is angiogenesis, or the formation of new blood vessels. Again MC are strategically located and are a rich source of angiogenic mediators. These include TNF, IL-8, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), which have all been implicated in the propagation of endothelial cell growth and function (61). In addition, MC release a number of products, including tryptase and matrix metalloproteinases (MMP), that degrade the stromal matrix to provide space for new vessel growth (62). Interestingly, MC have been identified as a source of the CC chemokine, I-309 (63). I-309 is chemoattractant for many cells types, and has recently been show to promote angiogenesis (64). Thus many factors released by MC may have multiple effects.

MC are also present in epithelial layers including the lung and the GI tract. Model sytems of food allergy and parasitic infection have implicated MC in regulating mucosal function and permeability (25). Histamine has been shown to increase ion secretion from colonic cells, and treatment with cyclooxygenase inhibitors has a similar effect (65). More recent studies have identified MC tryptase, via proteinase-activated receptor 2 (PAR-2), as having a role in mucosal dysregulation (66). Disruption of epithelial barrier function may also involve MC-derived histamine and prostaglandins, potentially through the activity of the radical, nitric oxide (NO)(67).

D. Mast cells in disease.

MC have a long association with immediate Type-I hypersensitivity responses that include allergic inflammatory conditions such as asthma. Asthma is a chronic disease of the airways with hallmark characteristics of bronchial hyperresponsiveness, inflammation and airway obstruction (19). Many cell types are involved in these response cascades, however, due to their resident tissue location (airway walls) and multifunctional mediators, MC are the initial effector cells (3). The existing paradigm for asthmatic inflammation consists of both an immediate-phase followed by a late-phase response (LPR)(19).

Activation of MC via specific antigen (allergen)-induced cross-linking of IgE induces immediate (1 to 30 min) degranulation and release of pre-formed and granule-derived mediators (3). Mediators such as histamine and leukotrienes are associated with the immediate response seen in tissues directly adjacent to MC. These targets and responses include histamine and leukotriene's causing bronchoconstiction via airway smooth muscle, and leukotrienes and prostaglandins causing increased venular permeability and vasodilation (19).

MC activation is also associated with the LPR seen in chronic allergic inflammation. Release of newly synthesized cytokines (eg. TNF, IL-5) results in an increased influx of granulocytic cells, including eosinophils (3). Release of chemokines, including MCP-1 and I-309 also induce recruitment of T-cells in addition to eosinophils (32,68). Thus, a milieu of activated inflammatory cells is drawn to the airways causing further hyperresponsiveness and airway tissue damage.

E. Regulators of mast cell responsiveness.

MC involvement in physiology and pathology, combined with their plethora of potent mediators has stimulated increased study on ways of controlling MC function and thus MC-related disease. Several inhibitor receptors expressed in MC have been identified, including the Ig-like molecules FcγRIIB, gp49BI, and KIR and the C-type lectin containing Ly49 and mast cell function-associated antigen (MAFA), though orthologues in human MC have been incompletely described (69). These receptors collectively are known as the inhibitory receptor superfamily (IRS) and all contain at least one conserved immunoreceptor tyrosine-based inhibition

motif (ITIM). These ITIM sites differentially recruit phosphates enzymes, and thus regulate the phosphorylation events involved in MC signaling, particularly through FcεRI. FcγRIIB is the most-studied receptor in this class and activation of this receptor by IgG binding is though to be one mechanism involved in the effects of antigen specific immunotherapy (37).

There are also several drugs that are known inhibitors of MC responsiveness, including β-agonists (eg. salbutamol, salmeterol), prostaglandin analogues (PGE_1 and misoprostol), corticosteroids (eg. dexamethasone) and sodium cromoglycate (SCG). As a group these drugs have broad-spectrum effects on MC function including inhibition of granule mediator secretion and decreased cytokine production (3).

Several cytokines can also moderate MC responsiveness in addition to their roles already outlined in MC development, including IL-10, transforming growth factor-β (TGF-β) and interferon-γ (IFN-γ)(33,70,71). Previously we have shown that IFN-γ inhibits histamine and TNF release from rat PMC (33), and adhesion in a rat MC line (72) in addition IFN-γ treatment modulates MC adhesive events. Several of these functions of IFN-γ have been associated with the increased expression of nitric oxide synthase and release of NO. Indeed, several studies have shown that NO has potent inhibitory activity in MC responsiveness including granule mediator release and cytokine production (73).

II. Nitric oxide

A. General Properties.

NO is one of the smallest bioactive molecules in nature with a size of only 30 Da. The union of one nitrogen and one oxygen means that NO has an unpaired electron in its outer valence orbit, thus making it a radical. First identified as an industrial pollutant and considered highly toxic, it has since evolved along a remarkable course being used as a preservative and therapeutic for cardiac dysfunction (74).

NO displays unique chemical characteristics including an inability to react with itself or to form chemical hydrates with water (H_2O)(74). It reacts in a similar fashion as molecular oxygen, and freely diffuses across membranes and other

cellular components. Recently, these properties have been further defined as hemoglobin has the *in-vivo* capability to not only bind oxygen and carbon dioxide but also NO (75).

NO plays crucial, diverse, and often divergent (homeostatic/pathologic) roles in vascular, neuronal and immunological systems (76).

B. Nitric oxide chemistry.

The pleiotropic effects of NO are hypothesized to be cause by its distinctive chemical properties. As such, NO has a large and diverse spectrum of partners (Fig. 1.3). Recently, attempts have been made to broadly classify NO effects into "direct" and "indirect" categories (77).

Direct effects are due to NO binding directly to a target molecule. Such interactions are generalized to happen under relatively low concentrations of NO found under physiological conditions, though some exceptions doubtlessly exist. Likely the best characterized of these interactions is NO binding to transition-metal containing proteins. The best studied of these metal nitrosyl complexes is the interaction of NO with the iron heme on guanylate cyclase, resulting in the formation of cGMP with resulting regulatory effects on vascular tone (78). NO can also directly interact with the high-valence free radicals of oxygen (alkoxyl, $RO^{\cdot}$ or alkyl $ROO^{\cdot}$)(79). These oxygen radicals have been implicated in cell damage due to lipid peroxidation (79). As such, NO inhibits these reactive mechanisms and plays an anti-inflammatory role (Fig. 1.3).

The indirect effects of NO are due to higher order nitrogen oxides (NO_x) formed by the interaction of NO with molecular or radical oxygen molecules often when high levels of NO are expressed such as in inflammatory states (77). The most prevalent of these species are dinitrogen trioxide (N_2O_3) and peroxynitrite ($ONOO^-$) which cause nitrosative and oxidative stress respectively (80). These molecules are much more powerful oxidants than NO alone, and react with a broad spectrum of substrates including thiols, esters, guanosine, carbohydrates, lipids and DNA (81). Indeed, much study has focused on the capability of $ONOO^-$ to cause the nitration of phenol containing molecules such as tyrosine (82). The formation of 3-nitrotyrosine (3-NT) has been shown to have diverse effects on signaling pathway

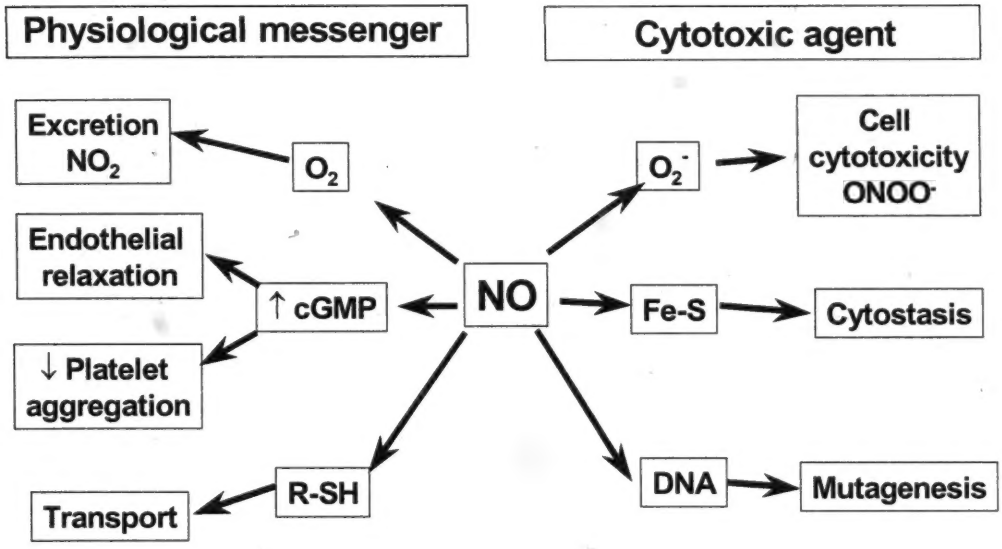


Fig. 1.3 Schematic representation of nitric oxide (NO) interactions in roles as a physiological messenger or a cytotoxic agent. NO mediates several of its well known signaling effects by binding the heme of guanylate cyclase, increasing the production of cGMP. NO can also interact with oxygen to form nitrite, which can be excreted. NO reacts with thiols (R-SH) to for nitrosothiols than can serve as a storage/transport site for NO. Under conditions of high NO formation, NO may interact with superoxide (O_2^-) to form the more reactive species, peroxynitrite ($ONOO^-$). NO can inhibit enzyme activity by reacting with metal containing functional groups. NO can also deaminate DNA resulting in mutagenic changes.

and released mediators by changing the active properties of these proteins (83). Such mechanisms have been implicated in a proposed, evolutionarily ancient signaling mechanism based on nitrogen instead of phosphate (phosphorylation)(84).

Further indirect effects are due to the interaction of NO with thiols and glutathione. The formation of these S-nitrothiols are thought to provide a stable storage platform for reactive nitrogen species (RNS), extending its biological half-life (85). Such S-nitrothiols have been shown to modulate MMP activity in airway models of inflammation (86).

C. Nitric oxide synthase.

1. General.

NO is formed by nitric oxide synthase (NOS; EC 1.14.13.39) in two discrete steps involving the conversion of L-arginine, to N-hydroxy-L-arginine, to L-citrulline with NO formed as a byproduct (76). NOS activity is multifactoral requiring numerous cofactors including the substrate; L-arginine, O_2, an electron donor; NADPH, heme, FAD, FMN, calmodulin and tetrahydrobiopterin (BH_4). The presence of two flavin groups is unique within human enzymatic systems (Fig. 1.4) (87). Three distinct isoforms have been isolated, initially named for the tissue of discovery, neuronal (nNOS), inducible (iNOS) and endothelial (eNOS)(88). NOS is active only in homodimeric conformation, with the indiviual subunits having molecular masses of 155 kDa (nNOS), 130 kDa (iNOS) and 135 kDa (eNOS)(Fig. 1.4). Catalytically it has been useful to distinguish between isoforms based on their activation dependency on Ca^{2+}. Both nNOS and eNOS are dependent on a Ca^{2+} signal in order to bind calmodulin and produce NO. Conversely, iNOS has robust calmodulin binding and Ca^{2+} is not required (76). As a general statement, nNOS and eNOS produce low amounts of NO (pM) while iNOS produces large amounts (nM)(76).

2. Structure and function.

On the basis of overall structure all NOS isoforms consist of a C-terminal reductase domain that contains the flavin moieties (FAD, FMN). By analogy this domain has structural homology similar to cytochrome P450 reductase, and it has been shown by mutational analysis that the flavins act as the initial e^- acceptors (89).

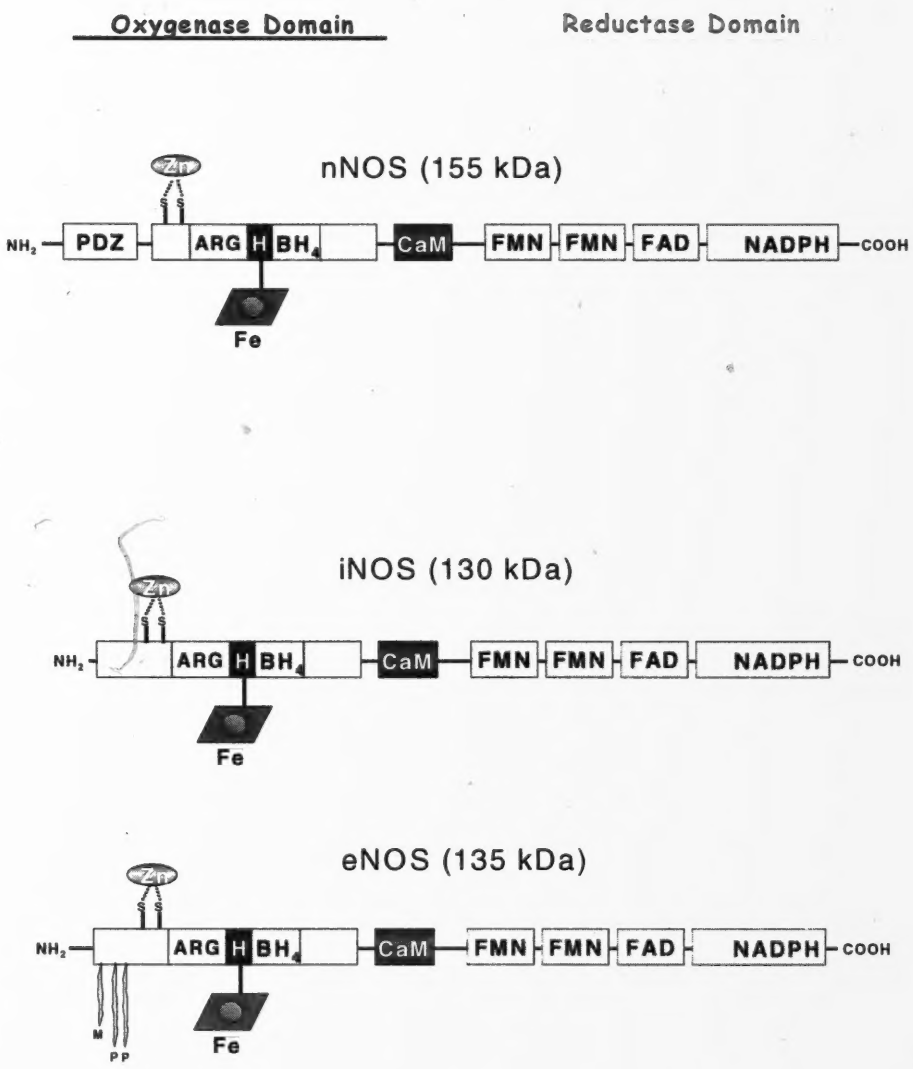


Fig. 1.4 Schematic of the monomeric structure of nitric oxide synthase (NOS). Oxygenase and reductase domains are represented by green and blue boxes respectively. The arginine (Arg), tetrahydrobiopterin (BH_4), flavin (FMN, FAD) and nicotinamide-adenine dinucleotide phosphate (NADPH) binding regions are denoted within the structure. The PDZ binding region in neuronal NOS (nNOS) and myristoylation (M) and palmitoylation (P) sites in endothelial NOS (eNOS) are shown. Inducible NOS (iNOS). Approximate molecular weights are also noted.

A calmodulin binding domain lies in the center pocket of the enzyme. As calmodulin is a protein, active NOS can actually be considered a tetramer of 2 NOS and 2 calmodulin (76). The N-terminus consists of the oxygenase domain enclosing the heme, O_2, BH_4 and arginine binding sites (Fig. 1.4). Spectroscopic studies have identified a cysteinyl-thiolate binding arm for the heme molecule and crystal structure study has identified the heme pocket as the putative catalytic site (90). The oxygenase domain has been described as a "baseball glove", with the pocket holding the arginine molecule (90).

The N-terminus also represents the region of most diversity amongst the NOS isoforms. nNOS contains a 300 amino acid PDZ-binding domain that is involved in targeting and binding interactions of nNOS. Such binding partners include the N-methyl-D-aspartate (NMDA) receptor in neuronal tissue, which allows direct linkage of a Ca^{2+} channel to nNOS (91). The eNOS isoforms exhibits a smaller N-terminal sequence that comprised three fatty acid acylation sites (myristoylation and palmitoylation) that are also critical for cellular targeting (Fig. 1.4) (92). Mutational analysis and expression has shown that dual acylation is required for the correct localization of eNOS to cellular membranes, inhibition of this modification has been shown to inhibit eNOS activity (92).

Electron flow, founded on P450 chemistry, proceeds from the NADPH donor, to FAD, then FMN to the heme region (87). Based on partial crystal structures and chimeric experiments, e^- transfer from FMN has been proposed to proceed to the heme group on the opposing monomer (87). In the presence of the e^- equivalents and O_2, arginine is hydroxylated and NO is formed. The role of BH_4 is unresolved, and may also be involved in e^- transfer (93). Additionally BH_4 is required to maintain the NOS dimer (94). Recent crystallography also identified a previously undetected zinc molecule at the dimer interface, though its precise role also remains undefined (Fig. 1.5) (95).

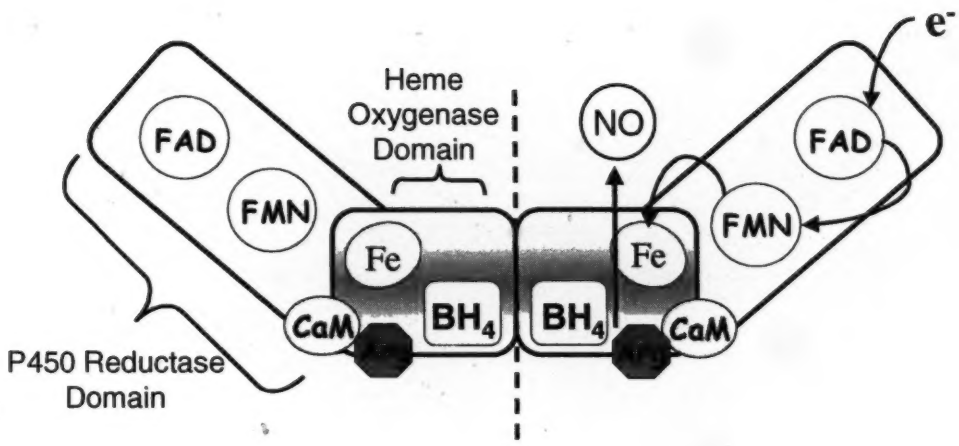


Fig. 1.5 Schematic of nitric oxide formation and electron (e^-) flow through nitric oxide synthase. e^- are donated by nicotinamide-adenine dinucleotide phosphate (NADPH) to the flavin-adenine dinucleotide (FAD) and them flavin mononucleotide (FMN) binding regions in the reductase domain. They then interact with the heme iron and tetrahydrobiopterin (BH_4) at the catalytic site to produce nitric oxide. Electron flow from the reductase domain to the oxygenase domain requires arginine, BH_4 and calmodulin.

3. Expression and post-translational regulation.

a) Transcriptional regulation. The initial studies investigating NOS involved delineating mRNA expression patterns. In some way this was unfortunate as it lead to the classification of NOS into isoforms that were "constitutively" expressed (nNOS/eNOS) or "induced" (iNOS) (96). This paradigm has cast a long shadow on the field despite a growing number of exceptions (97). Indeed, all isoforms have differing capacity for inducibility, and all have situations where they are constitutively expressed (Fig. 1.6).

nNOS mRNA was first characterized in neuronal tissue, though many non-neuronal sources have been identified (96). The human nNOS gene and promoter is located on chromosome 12 and is the largest of all the NOS's at 10 kb. Initial Northern blot studies screening multiple tissues showed numerous splice variants of nNOS particularly in the testis (98). These variants lack exon-2 which encodes for the PDZ domain, implying different localization than the full length variant (99). Heterologous expression has shown that these splice variants have NOS activity (99). nNOS mRNA expression can also be regulated by a number of physiological stimuli, including hypoxia, electrical stimulation and hormones (100). Interestingly, numerous inflammatory cytokines (eg. TNF, IFN-γ) down-regulate nNOS in many cell systems (101).

iNOS was first identified in activated macrophages. The human iNOS gene is located on chromosome 17 and is substantially smaller than nNOS at 4.8 kb (96). As its name implies, iNOS is upregulated in activated immune cells and there are many classical activators of iNOS including IL-1β, LPS, IFN-γ, and TNF (96). Of note is the apparent difficulty in detecting iNOS mRNA following similar treatments in human immune cells, though iNOS is also constitutively expressed in human hepatocytes (102). Several splice variants of iNOS have been identified, though so far their existence is limited to human cells (103). Most of the variants represent large splices within the heme domain and thus are thought to be catalytically inactive (104). Interestingly, studies on iNOS protein expression in multiple human tissues has identified truncated iNOS proteins comparable to translational products of these splice variants (105).

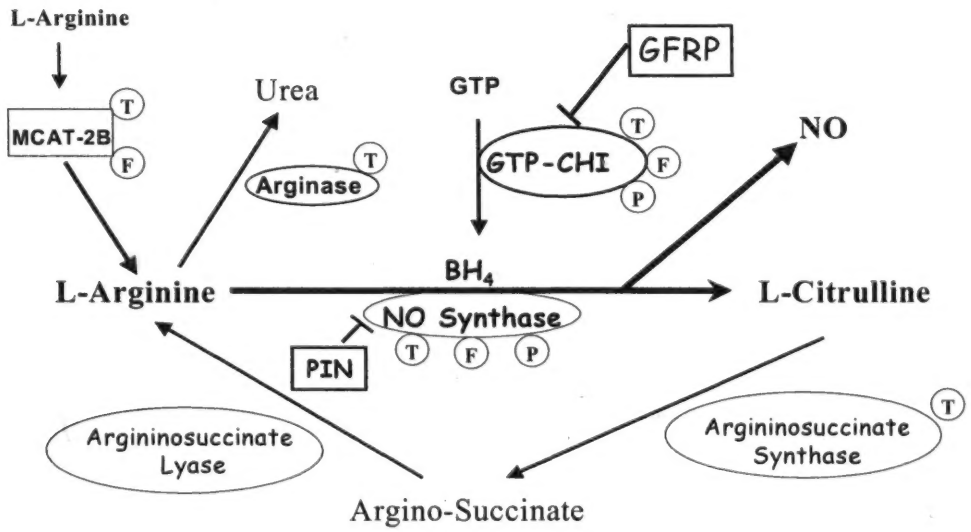


Fig. 1.6 Regulatory pathways of nitric oxide production. Enzymes involved in the process are in ovals, transporters are represented as rectangles. (Tetrahydrobiopterin, BH_4; GTP-CHI feedback regulatory protein, GFRP; GTP cyclohydrolase I, GTP-CHI; mouse cationic amino acid transporter, MCAT-2B; protein inhibitor of NOS, PIN). Known regulatory effects are noted as, "T" for transcriptional regulation, "F" for functional changes, and "P" for phosphorylation.

The human eNOS gene is located on chromosome 7 and is similar in size to the iNOS gene. No known splice variants of eNOS have been identified, though some polymorphisms may be associated with disease (106). The eNOS promoter is complex, and contains binding sites for many transcription factors including AP-1 and NF-κB (107). As such, multiple stimuli have also been shown to regulate eNOS expression including IFN-γ, shear stress and estrogens (108).

b) Cofactor regulation. As NOS activity is a multi-factor process and given that NOS has obligate requirements for the substrate arginine as well as BH_4, these factors also play a crucial and dynamic role in regulating NO formation (Fig. 1.6).

i) Arginine. NOS enzymes have obligate requirements for the substrate arginine, which is the requisite and sole nitrogen donor (109). Cellular arginine levels are dependent on a variety of synthesis and transport mechanisms, including uptake from extracellular fluid, intracellular protein breakdown, or endogenous synthesis (109).

Transport of arginine often increases in cells that have been activated to produce NO (110). This increased transport is due to the cationic amino acid transporter (CAT-2B), resulting in preferential uptake of L-arginine, though L-ornithine and L-lysine can also compete for binding sites (111). Interestingly, in some cell types it has been noted that intracellular levels of arginine are adequate to support NOS activity, even in the absence of extracellular arginine (110), though this may effect the long-term production of NO by iNOS. Indeed, sites of inflammation create an environment where extracellular arginine levels are not sufficient for NO production. However, several groups have shown that many cell types are able to recycle L-citrulline back to arginine through the activity of argininosuccinate synthase (AS) and argininosuccinate lyase (AL)(Fig.1.6)(112). Studies in LPS activated mouse macrophages showed that AS mRNA and protein were induced concordantly with iNOS, maintaining NO production in the absence of exogenous arginine (113). Interestingly, AL levels are not effected by this treatment, and AS levels are thus rate-limiting (114).

Other mechanisms also exist to control arginine availability. The enzyme arginase, Type I (constitutive) or Type II (inducible), catalyzes the breakdown of

arginine into urea and ornithine (Fig.1.6)(109). Type II arginase is expressed in mouse macrophages and cytokines that induce iNOS, such as IFN-γ, downregulate arginase activity, whereas the reverse is seen with IL-4 (115). Such an opposing mechanism has been recently proposed for NO regulation of T-cells (116).

ii)Tetrahydrobiopterin (BH_4). NOS enzymes also have a requirement for the cofactor BH_4 . Indeed, increases in NOS activity must be accompanied by a simultaneous increase in BH_4 levels, or NO production decreases (117). Considering the critical role played by BH_4 in NO synthesis, its precise role is unknown (118). In other enzymatic systems, BH_4 acts as an electron donor. However, reconstitution and stability assays have implicated BH_4 as an allosteric activator of NOS by maintaining the enzyme in its active dimerized state (119). Furthermore, BH_4 has been implicated in maintaining iNOS mRNA levels (120) and may also protect iNOS from inhibition by NO it produces (121). Clearly BH_4 is critical in catalyzing and maintaining NO production.

The majority of BH_4 is produced from *de novo* GTP by the activity of a complex pathway of enzymes under control of the rate-limiting enzyme in this pathway, GTP cyclohydrolase I (CHI) (Fig.1.6)(118). Most cells also maintain a minor capacity to recycle pterin molecules to form BH_4. The original paradigm was that increased iNOS induction was closely followed by an increase in GTP-CHI mRNA production, and presumably GTP-CHI protein translation (122). Thus BH_4 production was under transcriptional control through CHI. However, recent studies have shown post-translational regulatory mechanisms of CHI, including phosphorylation (123) and interaction with a recently cloned 9.5 kDa protein named GTP-CHI feedback regulatory protein (GFRP)(Fig.1.6) (124). GFRP binds CHI in the presence of BH4 and induces a conformational change that inactivates the enzyme. The expression pattern of CHI and GFRP are complex, do not always overlap and are often differentially regulated (125).

Levels of BH_4 have also been identified as a determining factor in the reactive radicals derived from NOS. At sub-saturating levels of BH_4 the NOS dimer can produce superoxide (O_2^-) or $ONOO^-$, rather than NO (126). This mechanism has been implicated in arteriosclerosis (127), where reconstitution or stabilization of

cellular BH_4 levels results in normalized NO production and increased vessel relaxation (128).

c) Localization and protein/protein interactions.

One of the most intense areas of study in recent years is the post-translational regulation of NOS. Owing to its important biological role and unique structural biology NOS must be tightly controlled to regulate its effects and this has resulted in a growing list of NOS interacting and regulating proteins (129).

i) Caveolin. The subcellular localization and control of eNOS has been the subject of intense investigation. One of the earliest defined eNOS characteristics was that it could be found in the particulate portion after subcellular fractionation (130). Cloning of the eNOS protein revealed that it had no transmembrane domain, and mutational analysis identified that the acylation of the N-terminus was responsible for its localization (92). Targeted yeast-two hybrid analysis revealed the binding of eNOS to caveolin, the major component of plasma membrane invaginations called caveolae (131). Caveolae are enriched in cholesterol and are known to sequester signaling molecules. Indeed, the majority of endothelial cell eNOS resides in caveolae (132). Interestingly, caveolae interactions with eNOS have been shown to inhibit NO formation, and microinjection of caveolin-like peptides also negates NO production from eNOS (133). The prevailing paradigm is activation of endothelial cells causes a Ca^{2+} influx that promotes calmodulin to bind to eNOS and concordant displacement of caveolin; this is known as the Calmodulin/caveolin switch (76). This model is supported by recent *in-vivo* work with caveolin deficient mice that shows increased vascular eNOS activity (134).

Evidence of caveolin interaction with other NOS isoforms is weaker, although nNOS interaction with caveolin-3 has been shown in skeletal muscle (135). This interaction also negatively regulates NO formation, though transfection studies have implied that the interaction is indirect and occurs through other proteins present in the dystrophin complex (135).

ii) Heat shock protein 90. Hsp90 is one of the most abundant cellular proteins comprising 1-2% of all cell constituents (136). Hsp90 and its yeast counterparts are crucial to proper protein folding and maturation (136). Deletion

and colocalization studies have revealed Hsp90 in sites other than the Golgi and ER and thus it may have other roles. Indeed, analysis of activated endothelial cells has shown that Hsp 90 tightly associates with eNOS which is correlated with increased NO formation (137). The precise mechanism is unknown, though Hsp90 may be able to stabilize the activated NOS homodimer (137). Further assays using purified proteins have shown that Hsp90 is an allosteric activator of eNOS, and studies in cell free systems revealed that Hsp90 may play a role in scaffolding and allowing many signaling proteins, including the kinase Akt, to interact in a large complex. (138) Of note is that in *Drosophila* Hsp90 is crucial for tyrosine kinase signaling though these results require further confirmation in vivo (139).

iii) Other NOS interacting proteins. Through the use of yeast two-hybrid techniques a growing complement of NOS interacting proteins have been identified.

Using the C-terminus of eNOS as "bait" a 34 kDa eNOS interacting protein (NOSIP) has been identified (140). Coimmunoprecipitation confirmed the interaction between eNOS and NOSIP, and immunohistochemistry documented the coexpression of these proteins in multiple tissues (141). NOSIP was originally thought to inhibit NOS activity, though recent transfection experiments revealed that NOSIP causes the translocation of eNOS to the cytoplasm, which may explain NOSIPs role in inhibition (140).

Another eNOS interacting protein called eNOS traffic inducer protein (NOSTRIN) has also been identified (142). NOSTRIN is unique in that it contains an SH3 domain, and as such it is potentially an adaptor protein involved in signaling (142). Overexpression of NOSTRIN in endothelial cells resulted in a redistribution of eNOS into vesicular structures in the cytoplasm, with concurrent inhibition of eNOS activity (142). Thus NOSTRIN and NOSIP appear to play a similar role in the trafficking of eNOS, though this role and specificity of action require further investigation.

Using nNOS as the target revealed an 89 a.a. interacting protein named protein inhibitor of NOS (PIN)(143). Mutational analysis initially identified PIN as an inhibitor of nNOS due to its ability to inhibit dimerization (143). Indeed, initial studies showed that PIN could decrease NO formation in transfected cells (144).

However, PIN also shares >90% homology with the light chain of the dynein complex. Dyneins are "molecular motors" that mobilize proteins and organelles towards the negative ends of microtubules (145). As such PIN has more recently been shown to interact with a variety of apparently unrelated proteins including myosin, IκB and the TGF-β receptor (146). Furthermore, PIN has been shown to interact with all NOS isoforms (147). Thus, PIN may also be involved in binding and trafficking of NOS and may provide a signaling link between NOS and the cytoskeleton.

d) Phosphorylation.

Phosphorylation is a critical posttranslation regulatory mechanism of diverse cellular proteins. Preliminary studies revealed that all NOS isoforms could be isolated as phospho-proteins from cell culture systems (148). This was of particular interest as it implicated a link between NOS and multiple cell signaling pathways.

eNOS regulation by phosphorylation has received the most attention. Studies using pharmacological inhibitors of PI-3K, PKC and other signaling molecules identified eNOS as a target for phosphoregulation of NO production (149). One of the significant downstream targets from PI-3K is the protein kinase, Akt. Akt has been shown in-vitro and in-vivo to phosphorylate human eNOS at serine 1177 (Ser^{1177})(150). Ser^{1177} resides at the C-terminal end of eNOS, and phosphorylation of this site leads to a 15-fold increase in NO formation (151). The precise mechanism is unclear, but it is believed that phosphorylation contributes to a conformational change that is conducive to increase e^- flow in the NOS dimer (151). Mutation of Ser^{1177} results in a decrease in NO formation, though this inhibition is incomplete. Thus, other cellular signaling cascades, including AMP-activated protein kinase (AMPK) and calmodulin kinase (CaMKII), may also be involved in regulating eNOS activity (152).

Human eNOS can also be phosphorylated at threonine 495 (Thr^{495}). Phosphorylation at Thr^{495} appears to be constitutive in all cell types so far studied (153). Cell free analysis reveals that phosphorylated Thr^{495} has an inhibitory tone on eNOS activity (154). Indeed, Thr^{495} is present in the CaM-binding domain and likely inhibits Calmodulin association. PKC seems to be important for maintaining

Thr^{495} in a phosphorylated state (155). Interestingly, various studies have shown a complex and enigmatic eNOS phosphorylation pattern at Thr^{495} and Ser^{1177} (149). Thus, eNOS activity and NO formation can be agonist-specific, and represent a particular balance between phosphorylation states.

4. Nitric oxide and mast cells.

Since the initial discovery of NO it has been implicated in the regulation of MC, and because MC mediators contribute to inflammatory responses, control of MC function via NO may be of benefit in many inflammatory diseases. Indeed, inhibition of NO synthesis with NOS inhibitors (L-NMMA) enhances MC degranulation in *in-vitro* and *in-vivo* systems (156,157). However, interpretation of these results has been difficult, as early studies employed impure MC populations (<80% MC)(156). In addition NO derived from contaminating cells has been shown to be an exogenous source of NO that regulated MC responsiveness, as such it was difficult to attribute NO effects in MC to exogenous or endogenous sources (156). Compounding this difficulty is the direct inhibition of MC serotonin and TNF release by pretreatment with exogenous NO donors (158,159).

The ability of NO to regulate mediator release from MC may be physiologically relevant. Indeed, MC-derived NO has been shown recently to be a critical regulatory element in controlling vascular permeability with inhibition of NO synthesis causing MC degranulation, an increase in microvascular permeability, and leukocyte infiltration (67). In addition, there is a growing association between MC NO and vascular dysfunction such as reperfusion injury (160). This concept is indirectly supported by experiments with MC stabilizing compounds that mimic the effect of NO administration causing reduction of vascular dysfunction (67).

There is increasing evidence of a physiological role for NO in regulating MC activity, however the mechanism(s) employed by NO remain unclear. Initial studies from our group have shown that endogenous NO modulates MC adhesion in both human and rodent systems (72,161). As adhesion is required for MC activation, this may be a potential mechanism of NO action (162). In addition, nitrosylation of cellular proteins including calpain, a critical protease in cell adhesion, and small G

proteins including Rac have been shown as potential targets in MC and other systems (161,163).

III. Central hypothesis and aims.

The release of MC mediators such as histamine and leukotrienes by exocytotic or secretory mechanisms is known to both initiate and propagate immune responses and chronic activation can result in severe inflammatory diseases such as asthma. Inhibition of MC activation is thus a key component in treating asthma. NO has been shown to downregulate MC reactivity by influencing critical cell pathways (164). However the topic of MC-derived NO is controversial, with NO being proposed to have both pro- and anti-inflammatory roles in MC function (165). In addition, until recently, some doubt remained as to the capacity of MC to endogenously produce NOS and NO. Thus, an understanding of molecular mechanisms of production and regulation of NO in MC will be important in developing novel therapeutics to control MC responses.

The central hypothesis is that MC (both rodent and human) are a source of NO produced by the enzyme NOS. We further hypothesize that NO production and the outcome effects (pro- or anti-inflammatory) of NO on MC are critically dependent on availability of cofactors and the influence of NOS/protein interactions.

Specific objectives:

1. **To characterize NOS expression and localization in rodent and human MC populations.** The expression and relative contribution of the three NOS isoforms (nNOS, iNOS, and eNOS) to MC NO production is unknown. RT-PCR and western blot will be used to identified NOS mRNA and protein expression. In addition, regulation of NOS expression by a variety of stimuli, including cytokines such as IFN-γ (a known inhibitor of MC function) will also be studied. To investigate the spatial cellular distribution of NOS, confocal microscopy will be employed. Subcellular fractionation experiments will be employed to confirm the microscopy results.
2. **To determine NO formation in MC**: The reactive nature of NO precludes its direct detection, particularly when expressed at low levels as in MC. A variety

of techniques to detect stable breakdown products of NO (Griess assay), determine NOS activity (Radioactive citrulline assay) and a novel, live-cell confocal assay to determine NO production in MC using the NO-sensitive fluorescent probe, diaminofluorescein (DAF), will be employed.

3. **To establish the effects of the availability of critical cofactors in modulating MC NOS activity:** NOS has obligate requirements for BH_4 as both an allosteric molecule and electron transfer substrate. BH_4 is produced *de novo* by the enzyme CHI. CHI mRNA and protein expression in both rat and human MC will be determined. Pharmacological inhibition of CHI activity and effects on NO formation will be determined.
4. **To determine the activation and post-translational regulatory pathways of NOS in MC:** Given the complex regulation of NOS, investigations into regulation of phosphorylation state will be undertaken using western blot techniques. In addition, identification and control of NOS interacting proteins will be defined by gene array and other studies.
5. **To define the roles and effects that endogenous NO plays in MC function:** The effects of modulating NO on various MC responses including degranulation, lipid-mediator release and cytokine/chemokine formation will be measured.

References

1. Wedemeyer, J., Tsai, M., and Galli, S.J. 2000. Roles of mast cells and basophils in innate and acquired immunity. *Curr. Opin. Immunol.* 12:624-631.

2. Benoist, C. and Mathis, D. 2002. Mast cells in autoimmune disease. *Nature* 420:875-878.

3. Metcalfe, D.D., Baram, D., and Mekori, Y.A. 1997. Mast cells. *Physiol. Rev.* 77:1033-1079.

4. Eguchi, M. 1991. Comparative electron microscopy of basophils and mast cells, in vivo and in vitro. *Electron Microsc. Rev* 4:293-318.

5. Dvorak, A.M. and Kissell, S. 1991. Granule changes of human skin mast cells characteristic of piecemeal degranulation and associated with recovery during wound healing in situ. *J. Leukoc. Biol.* 49:197-210.

6. Mwamtemi, H.H., Koike, K., Kinoshita, T., Ito, S., Ishida, S., Nakazawa, Y., Kurokawa, Y., Shinozaki, K., Sakashita, K., Takeuchi, K., Shiohara, M., Kamijo, T., Yasui, Y., Ishiguro, A., Kawano, Y., Kitano, K., Miyazaki, H., Kato, T., Sakuma, S., and Komiyama, A. 2001. An increase in circulating mast cell colony-forming cells in asthma. *J. Immunol.* 166:4672-4677.

7. Agis, H., Willheim, M., Sperr, W.R., Wilfing, A., Kromer, E., Kabrna, E., Spanblochl, E., Strobl, H., Geissler, K., Spittler, A. 1993. Monocytes do not make mast cells when cultured in the presence of SCF. Characterization of the circulating mast cell progenitor as a c-kit+, CD34+, Ly-, CD14-, CD17-, colony-forming cell. *J. Immunol.* 151:4221-4227.

8. Andrews, R.G., Briddell, R.A., Appelbaum, F.R., and McNiece, I.K. 1994. Stimulation of hematopoiesis in vivo by stem cell factor. *Curr. Opin. Hematol.* 1:187-196.

9. Alizadeh, H. and Murrell, K.D. 1984. The intestinal mast cell response to Trichinella spiralis infection in mast cell-deficient W/W^v mice. *J. Parasitol.* 70:767-773.

10. Kaartinen, M., Penttila, A., and Kovanen, P.T. 1996. Mast cells accompany microvessels in human coronary atheromas: implications for intimal neovascularization and hemorrhage. *Atherosclerosis* 123:123-131.

11. Bauer, O. and Razin, E. 2000. Mast cell-nerve interactions. *News Physiol. Sci.* 15:213-218.

12. Mayrhofer, G. 1980. Fixation and staining of granules in mucosal mast cells and intraepithelial lymphocytes in the rat jejunum, with special reference to the relationship between the acid glycosaminoglycans in the two cell types. *Histochem. J.* 12:513-526.

13. Strobel, S., Miller, H.R., and Ferguson, A. 1981. Human intestinal mucosal mast cells: evaluation of fixation and staining techniques. *J. Clin. Pathol.* 34:851-858.

14. Irani, A.M., Bradford, T.R., Kepley, C.L., Schechter, N.M., and Schwartz, L.B. 1989. Detection of MC^T and MC^{TC} types of human mast cells by immunohistochemistry using new monoclonal anti-tryptase and anti-chymase antibodies. *J. Histochem. Cytochem.* 37:1509-1515.

15. Beil, W.J., Schulz, M., and Wefelmeyer, U. 2000. Mast cell granule composition and tissue location--a close correlation. *Histol. Histopathol.* 15:937-946.

16. Li, L., Meng, X.W., and Krilis, S.A. 1996. Mast cells expressing chymase but not tryptase can be derived by culturing human progenitors in conditioned medium obtained from a human mastocytosis cell strain with c-kit ligand. *J. Immunol.* 156:4839-4844.

17. Li, L. and Krilis, S.A. 1999. Mast-cell growth and differentiation. *Allergy* 54:306-312.

18. Ahn, K., Takai, S., Pawankar, R., Kuramasu, A., Ohtsu, H., Kempuraj, D., Tomita, H., Iida, M., Matsumoto, K., Akasawa, A., Miyazaki, M., and Saito, H. 2000. Regulation of chymase production in human mast cell progenitors. *J. Allergy Clin. Immunol.* 106:321-328.

19. Brightling, C.E., Bradding, P., Pavord, I.D., and Wardlaw, A.J. 2003. New insights into the role of the mast cell in asthma. *Clin. Exp. Allergy* 33:550-556.

20. Supajatura, V., Ushio, H., Nakao, A., Akira, S., Okumura, K., Ra, C., and Ogawa, H. 2002. Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity. *J. Clin. Invest.* 109:1351-1359.

21. Hallgren, J., Estrada, S., Karlson, U., Alving, K., and Pejler, G. 2001. Heparin antagonists are potent inhibitors of mast cell tryptase. *Biochemistry* 40:7342-7349.

22. Alfonso, A., Cabado, A.G., Vieytes, M.R., and Botana, L.M. 2000. Calcium-pH crosstalks in rat mast cells: cytosolic alkalinization, but not intracellular calcium release, is a sufficient signal for degranulation. *Br. J. Pharmacol.* 130:1809-1816.

23. Miller, H.R. and Pemberton, A.D. 2002. Tissue-specific expression of mast cell granule serine proteinases and their role in inflammation in the lung and gut. *Immunology* 105:375-390.

24. Hallgren, J., Spillmann, D., and Pejler, G. 2001. Structural requirements and mechanism for heparin-induced activation of a recombinant mouse mast cell tryptase, mouse mast cell protease-6: formation of active tryptase monomers in the presence of low molecular weight heparin. *J. Biol. Chem.* 276:42774-42781.

25. Yu, L.C. and Perdue, M.H. 2001. Role of mast cells in intestinal mucosal function: studies in models of hypersensitivity and stress. *Immunol. Rev.* 179:61-73.

26. Scott, K.F., Bryant, K.J., and Bidgood, M.J. 1999. Functional coupling and differential regulation of the phospholipase A2-cyclooxygenase pathways in inflammation. *J. Leukoc. Biol.* 66:535-541.

27. Peters-Golden, M. and Brock, T.G. 2001. Intracellular compartmentalization of leukotriene synthesis: unexpected nuclear secrets. *FEBS Lett.* 487:323-326.

28. Brock, T.G., Paine, R., III, and Peters-Golden, M. 1994. Localization of 5-lipoxygenase to the nucleus of unstimulated rat basophilic leukemia cells. *J. Biol. Chem.* 269:22059-22066.

29. O'Byrne, P.M. 1997. Leukotrienes in the pathogenesis of asthma. *Chest* 111:27S-34S.

30. Hsieh, F.H., Lam, B.K., Penrose, J.F., Austen, K.F., and Boyce, J.A. 2001. T helper cell type 2 cytokines coordinately regulate immunoglobulin E-dependent cysteinyl leukotriene production by human cord blood-derived mast cells: profound induction of leukotriene C(4) synthase expression by interleukin 4. *J. Exp. Med.* 193:123-133.

31. Kawakami, T. and Galli, S.J. 2002. Regulation of mast-cell and basophil function and survival by IgE. *Nat. Rev. Immunol.* 2:773-786.

32. Selvan, R.S., Butterfield, J.H., and Krangel, M.S. 1994. Expression of multiple chemokine genes by a human mast cell leukemia. *J. Biol. Chem.* 269:13893-13898.

33. Bissonnette, E.Y., Chin, B., and Befus, A.D. 1995. Interferons differentially regulate histamine and TNF-alpha in rat intestinal mucosal mast cells. *Immunology* 86:12-17.

34. Reischl, I.G., Coward, W.R., and Church, M.K. 1999. Molecular consequences of human mast cell activation following immunoglobulin E-high-affinity immunoglobulin E receptor (IgE- FcepsilonRI) interaction. *Biochem. Pharmacol.* 58:1841-1850.

35. Shefler, I., Taube, Z., Medalia, O., and Sagi-Eisenberg, R. 1998. Basic secretagogues activate protein tyrosine phosphorylation and release of arachidonic acid in mast cells via a novel protein kinase C and phosphatidylinositol 3-kinase-dependent mechanism. *Eur. J. Immunol.* 28:3468-3478.

36. Supajatura, V., Ushio, H., Nakao, A., Okumura, K., Ra, C., and Ogawa, H. 2001. Protective roles of mast cells against enterobacterial infection are mediated by Toll-like receptor 4. *J. Immunol.* 167:2250-2256.

37. Tkaczyk, C. and Gilfillan, A.M. 2001. Fc(varepsilon)Ri-dependent signaling pathways in human mast cells. *Clin. Immunol.* 99:198-210.

38. Heyman, B. 2000. Regulation of antibody responses via antibodies, complement, and Fc receptors. *Annu. Rev. Immunol.* 18:709-737.

39. Turner, H. and Kinet, J.P. 1999. Signalling through the high-affinity IgE receptor Fc epsilonRI. *Nature* 402:B24-B30.

40. Nechushtan, H., Leitges, M., Cohen, C., Kay, G., and Razin, E. 2000. Inhibition of degranulation and interleukin-6 production in mast cells derived from mice deficient in protein kinase Cbeta. *Blood* 95:1752-1757.

41. Letourneur, F., Hennecke, S., Demolliere, C., and Cosson, P. 1995. Steric masking of a dilysine endoplasmic reticulum retention motif during assembly of the human high affinity receptor for immunoglobulin E. *J. Cell. Biol.* 129:971-978.

42. Kawakami, Y., Kitaura, J., Satterthwaite, A.B., Kato, R.M., Asai, K., Hartman, S.E., Maeda-Yamamoto, M., Lowell, C.A., Rawlings, D.J., Witte,

O.N., and Kawakami, T. 2000. Redundant and opposing functions of two tyrosine kinases, Btk and Lyn, in mast cell activation. *J. Immunol.* 165:1210-1219.

43. Zhang, J., Berenstein, E.H., Evans, R.L., and Siraganian, R.P. 1996. Transfection of Syk protein tyrosine kinase reconstitutes high affinity IgE receptor-mediated degranulation in a Syk-negative variant of rat basophilic leukemia RBL-2H3 cells. *J. Exp. Med.* 184:71-79.

44. Vosseller, K., Stella, G., Yee, N.S., and Besmer, P. 1997. c-kit receptor signaling through its phosphatidylinositide-3'-kinase-binding site and protein kinase C: role in mast cell enhancement of degranulation, adhesion, and membrane ruffling. *Mol. Biol. Cell* 8:909-922.

45. Manetz, T.S., Gonzalez-Espinosa, C., Arudchandran, R., Xirasagar, S., Tybulewicz, V., and Rivera, J. 2001. Vav1 regulates phospholipase cgamma activation and calcium responses in mast cells. *Mol. Cell. Biol.* 21:3763-3774.

46. Wen, R., Jou, S.T., Chen, Y., Hoffmeyer, A., and Wang, D. 2002. Phospholipase C gamma 2 is essential for specific functions of Fc epsilon R and Fc gamma R. *J. Immunol.* 169:6743-6752.

47. Senyshyn, J., Baumgartner, R.A., and Beaven, M.A. 1998. Quercetin sensitizes RBL-2H3 cells to polybasic mast cell secretagogues through increased expression of Gi GTP-binding proteins linked to a phospholipase C signaling pathway. *J. Immunol.* 160:5136-5144.

48. Chahdi, A., Mousli, M., and Landry, Y. 1998. Substance P-related inhibitors of mast cell exocytosis act on G- proteins or on the cell surface. *Eur. J. Pharmacol.* 341:329-335.

49. Befus, A.D., Mowat, C., Gilchrist, M., Hu, J., Solomon, S., and Bateman, A. 1999. Neutrophil defensins induce histamine secretion from mast cells: mechanisms of action. *J. Immunol.* 163:947-953.

50. Shefler, I., Seger, R., and Sagi-Eisenberg, R. 1999. Gi-mediated activation of mitogen-activated protein kinase (MAPK) pathway by receptor mimetic basic secretagogues of connective tissue- type mast cells: bifurcation of arachidonic acid-induced release upstream of MAPK. *J. Pharmacol. Exp. Ther.* 289:1654-1661.

51. Law, G.J., Northrop, A.J., and Mason, W.T. 1993. rab3-peptide stimulates exocytosis from mast cells via a pertussis toxin-sensitive mechanism. *FEBS Lett.* 333:56-60.

52. Aridor, M., Traub, L.M., and Sagi-Eisenberg, R. 1990. Exocytosis in mast cells by basic secretagogues: evidence for direct activation of GTP-binding proteins. *J. Cell Biol.* 111:909-917.

53. Maurer, M., Echtenacher, B., Hultner, L., Kollias, G., Mannel, D.N., Langley, K.E., and Galli, S.J. 1998. The c-kit ligand, stem cell factor, can enhance innate immunity through effects on mast cells. *J. Exp. Med.* 188:2343-2348.

54. Aderem, A. and Ulevitch, R.J. 2000. Toll-like receptors in the induction of the innate immune response. *Nature* 406:782-787.

55. Underhill, D.M. 2003. Toll-like receptors: networking for success. *Eur. J. Immunol* 33:1767-1775.

56. Varadaradjalou, S., Feger, F., Thieblemont, N., Hamouda, N.B., Pleau, J.M., Dy, M., and Arock, M. 2003. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells. *Eur. J. Immunol.* 33:899-906.

57. McCurdy, J.D., Olynych, T.J., Maher, L.H., and Marshall, J.S. 2003. Cutting edge: distinct Toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells. *J. Immunol.* 170:1625-1629.

58. Huttunen, M., Aalto, M.L., Harvima, R.J., Horsmanheimo, M., and Harvima, I.T. 2000. Alterations in mast cells showing tryptase and chymase activity in epithelializating and chronic wounds. *Exp. Dermatol.* 9:258-265.

59. Abe, M., Yokoyama, Y., Amano, H., Matsushima, Y., Kan, C., and Ishikawa, O. 2002. Effect of activated human mast cells and mast cell-derived mediators on proliferation, type I collagen production and glycosaminoglycans synthesis by human dermal fibroblasts. *Eur. J. Dermatol.* 12:340-346.

60. Lazaar, A.L., Plotnick, M.I., Kucich, U., Crichton, I., Lotfi, S., Das, S.K., Kane, S., Rosenbloom, J., Panettieri, R.A., Jr., Schechter, N.M., and Pure, E. 2002. Mast cell chymase modifies cell-matrix interactions and inhibits mitogen-induced proliferation of human airway smooth muscle cells. *J. Immunol.* 169:1014-1020.

61. Boesiger, J., Tsai, M., Maurer, M., Yamaguchi, M., Brown, L.F., Claffey, K.P., Dvorak, H.F., and Galli, S.J. 1998. Mast cells can secrete vascular permeability factor/ vascular endothelial cell growth factor and exhibit enhanced release after immunoglobulin E-dependent upregulation of fc epsilon receptor I expression. *J. Exp. Med.* 188:1135-1145.

62. Ribatti, D., Vacca, A., Nico, B., Crivellato, E., Roncali, L., and Dammacco, F. 2001. The role of mast cells in tumour angiogenesis. *Br. J. Haematol.* 115:514-521.

63. Nakajima, T., Inagaki, N., Tanaka, H., Tanaka, A., Yoshikawa, M., Tamari, M., Hasegawa, K., Matsumoto, K., Tachimoto, H., Ebisawa, M., Tsujimoto, G., Matsuda, H., Nagai, H., and Saito, H. 2002. Marked increase in CC chemokine gene expression in both human and mouse mast cell transcriptomes following Fcepsilon receptor I cross-linking: an interspecies comparison. *Blood* 100:3861-3868.

64. Bernardini, G., Spinetti, G., Ribatti, D., Camarda, G., Morbidelli, L., Ziche, M., Santoni, A., Capogrossi, M.C., and Napolitano, M. 2000. I-309 binds to

and activates endothelial cell functions and acts as an angiogenic molecule in vivo. *Blood* 96:4039-4045.

65. Crowe, S.E., Luthra, G.K., and Perdue, M.H. 1997. Mast cell mediated ion transport in intestine from patients with and without inflammatory bowel disease. *Gut* 41:785-792.

66. Cenac, N., Coelho, A.M., Nguyen, C., Compton, S., Andrade-Gordon, P., MacNaughton, W.K., Wallace, J.L., Hollenberg, M.D., Bunnett, N.W., Garcia-Villar, R., Bueno, L., and Vergnolle, N. 2002. Induction of intestinal inflammation in mouse by activation of proteinase-activated receptor-2. *Am. J. Pathol.* 161:1903-1915.

67. Gaboury, J.P., Niu, X.F., and Kubes, P. 1996. Nitric oxide inhibits numerous features of mast cell-induced inflammation. *Circulation* 93:318-326.

68. Panina-Bordignon, P., Papi, A., Mariani, M., Di Lucia, P., Casoni, G., Bellettato, C., Buonsanti, C., Miotto, D., Mapp, C., Villa, A., Arrigoni, G., Fabbri, L.M., and Sinigaglia, F. 2001. The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. *J. Clin. Invest.* 107:1357-1364.

69. Ott, V.L. and Cambier, J.C. 2000. Activating and inhibitory signaling in mast cells: new opportunities for therapeutic intervention? *J. Allergy Clin. Immunol.* 106:429-440.

70. Lin, T.J. and Befus, A.D. 1997. Differential regulation of mast cell function by IL-10 and stem cell factor. *J. Immunol.* 159:4015-4023.

71. Bissonnette, E.Y., Enciso, J.A., and Befus, A.D. 1997. TGF-beta1 inhibits the release of histamine and tumor necrosis factor- alpha from mast cells through an autocrine pathway. *Am. J. Respir. Cell Mol. Biol.* 16:275-282.

72. Wills, F.L., Gilchrist, M., and Befus, A.D. 1999. Interferon-gamma regulates the interaction of RBL-2H3 cells with fibronectin through production of nitric oxide. *Immunology* 97:481-489.

73. Bidri, M., Feger, F., Varadaradjalou, S., Ben Hamouda, N., Guillosson, J.J., and Arock, M. 2001. Mast cells as a source and target for nitric oxide. *Int. Immunopharmacol.* 1:1543-1558.

74. Richter-Addo, G.B., Legzdins, P., and Burstyn, J. 2002. Introduction: nitric oxide chemistry. *Chem. Rev.* 102:857-860.

75. McMahon, T.J., Moon, R.E., Luschinger, B.P., Carraway, M.S., Stone, A.E., Stolp, B.W., Gow, A.J., Pawloski, J.R., Watke, P., Singel, D.J., Piantadosi, C.A., and Stamler, J.S. 2002. Nitric oxide in the human respiratory cycle. *Nat. Med.* 8:711-717.

76. Michel, T. and Feron, O. 1997. Nitric oxide synthases: which, where, how, and why? *J. Clin. Invest.* 100:2146-2152.

77. Grisham, M.B., Jourd'Heuil, D., and Wink, D.A. 1999. Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites:implications in inflammation. *Am. J. Physiol.* 276:G315-G321.

78. Friebe, A. and Koesling, D. 2003. Regulation of nitric oxide-sensitive guanylyl cyclase. *Circ. Res.* 93:96-105.

79. Chisolm, G.M., III, Hazen, S.L., Fox, P.L., and Cathcart, M.K. 1999. The oxidation of lipoproteins by monocytes-macrophages. Biochemical and biological mechanisms. *J. Biol. Chem.* 274:25959-25962.

80. Greenacre, S.A. and Ischiropoulos, H. 2001. Tyrosine nitration: localisation, quantification, consequences for protein function and signal transduction. *Free Radic. Res.* 34:541-581.

81. Maeda, H. and Akaike, T. 1998. Nitric oxide and oxygen radicals in infection, inflammation, and cancer. *Biochemistry (Mosc.)* 63:854-865.

82. Murphy, M.P., Packer, M.A., Scarlett, J.L., and Martin, S.W. 1998. Peroxynitrite: a biologically significant oxidant. *Gen. Pharmacol.* 31:179-186.

83. Hess, D.T., Matsumoto, A., Nudelman, R., and Stamler, J.S. 2001. S-nitrosylation: spectrum and specificity. *Nat. Cell Biol.* 3:E46-E49.

84. Stamler, J.S., Lamas, S., and Fang, F.C. 2001. Nitrosylation. the prototypic redox-based signaling mechanism. *Cell* 106:675-683.

85. Radi, R., Peluffo, G., Alvarez, M.N., Naviliat, M., and Cayota, A. 2001. Unraveling peroxynitrite formation in biological systems. *Free Radic. Biol. Med.* 30:463-488.

86. Okamoto, T., Valacchi, G., Gohil, K., Akaike, T., and van, d., V. 2002. S-nitrosothiols inhibit cytokine-mediated induction of matrix metalloproteinase-9 in airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 27:463-473.

87. Alderton, W.K., Cooper, C.E., and Knowles, R.G. 2001. Nitric oxide synthases: structure, function and inhibition. *Biochem. J.* 357:593-615.

88. Gorren, A.C. and Mayer, B. 1998. The versatile and complex enzymology of nitric oxide synthase. *Biochemistry (Mosc.)* 63:734-743.

89. Guan, Z.W., Kamatani, D., Kimura, S., and Iyanagi, T. 2003. Mechanistic studies on the intramolecular one-electron transfer between the two flavins in the human neuronal nitric-oxide synthase and inducible nitric-oxide synthase flavin domains. *J. Biol. Chem.* 278:30859-30868.

90. Li, H., Raman, C.S., Glaser, C.B., Blasko, E., Young, T.A., Parkinson, J.F., Whitlow, M., and Poulos, T.L. 1999. Crystal structures of zinc-free and -bound heme domain of human inducible nitric-oxide synthase. Implications

for dimer stability and comparison with endothelial nitric-oxide synthase. *J. Biol. Chem.* 274:21276-21284.

91. Rodrigo, J., Alonso, D., Fernandez, A.P., Serrano, J., Richart, A., Lopez, J.C., Santacana, M., Martinez-Murillo, R., Bentura, M.L., Ghiglione, M., and Uttenthal, L.O. 2001. Neuronal and inducible nitric oxide synthase expression and protein nitration in rat cerebellum after oxygen and glucose deprivation. *Brain Res.* 909:20-45.

92. Prabhakar, P., Cheng, V., and Michel, T. 2000. A chimeric transmembrane domain directs endothelial nitric-oxide synthase palmitoylation and targeting to plasmalemmal caveolae. *J. Biol. Chem.* 275:19416-19421.

93. Kotsonis, P., Frohlich, L.G., Shutenko, Z.V., Horejsi, R., Pfleiderer, W., and Schmidt, H.H. 2000. Allosteric regulation of neuronal nitric oxide synthase by tetrahydrobiopterin and suppression of auto-damaging superoxide. *Biochem. J.* 346 Pt 3:767-776.

94. Park, J.H., Na, H.J., Kwon, Y.G., Ha, K.S., Lee, S.J., Kim, C.K., Lee, K.S., Yoneyama, T., Hatakeyama, K., Kim, P.K., Billiar, T.R., and Kim, Y.M. 2002. Nitric oxide (NO) pretreatment increases cytokine-induced NO production in cultured rat hepatocytes by suppressing GTP cyclohydrolase I feedback inhibitory protein level and promoting inducible NO synthase dimerization. *J. Biol. Chem.* 277:47073-47079.

95. Raman, C.S., Li, H., Martasek, P., Kral, V., Masters, B.S., and Poulos, T.L. 1998. Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center. *Cell* 95:939-950.

96. Koshimura, K., Murakami, Y., Tanaka, J., and Kato, Y. 1998. Self-protection of PC12 cells by 6R-tetrahydrobiopterin from nitric oxide toxicity. *J. Neurosci. Res.* 54:664-672.

97. Forstermann, U., Boissel, J.P., and Kleinert, H. 1998. Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III). *FASEB J.* 12:773-790.

98. Wang, Y., Goligorsky, M.S., Lin, M., Wilcox, J.N., and Marsden, P.A. 1997. A novel, testis-specific mRNA transcript encoding an NH2-terminal truncated nitric-oxide synthase. *J. Biol. Chem.* 272:11392-11401.

99. Eliasson, M.J., Blackshaw, S., Schell, M.J., and Snyder, S.H. 1997. Neuronal nitric oxide synthase alternatively spliced forms: prominent functional localizations in the brain. *Proc. Natl. Acad. Sci. U. S. A* 94:3396-3401.

100. Scordalakes, E.M., Shetty, S.J., and Rissman, E.F. 2002. Roles of estrogen receptor alpha and androgen receptor in the regulation of neuronal nitric oxide synthase. *J. Comp. Neurol.* 453:336-344.

101. Alexander, B.T., Cockrell, K.L., Massey, M.B., Bennett, W.A., and Granger, J.P. 2002. Tumor necrosis factor-alpha-induced hypertension in pregnant rats results in decreased renal neuronal nitric oxide synthase expression. *Am. J. Hypertens.* 15:170-175.

102. Taylor, B.S., Alarcon, L.H., and Billiar, T.R. 1998. Inducible nitric oxide synthase in the liver: regulation and function. *Biochemistry (Mosc.)* 63:766-781.

103. Eissa, N.T., Yuan, J.W., Haggerty, C.M., Choo, E.K., Palmer, C.D., and Moss, J. 1998. Cloning and characterization of human inducible nitric oxide synthase splice variants: a domain, encoded by exons 8 and 9, is critical for dimerization. *Proc. Natl. Acad. Sci. U. S. A* 95:7625-7630.

104. Chu, S.C., Wu, H.P., Banks, T.C., Eissa, N.T., and Moss, J. 1995. Structural diversity in the 5'-untranslated region of cytokine- stimulated human inducible nitric oxide synthase mRNA. *J. Biol. Chem.* 270:10625-10630.

105. Park, C.S., Krishna, G., Ahn, M.S., Kang, J.H., Chung, W.G., Kim, D.J., Hwang, H.K., Lee, J.N., Paik, S.G., and Cha, Y.N. 2000. Differential and constitutive expression of neuronal, inducible, and endothelial nitric oxide synthase mRNAs and proteins in pathologically normal human tissues. *Nitric Oxide* 4:459-471.

106. Jachymova, M., Horky, K., Bultas, J., Kozich, V., Jindra, A., Peleska, J., and Martasek, P. 2001. Association of the Glu298Asp polymorphism in the endothelial nitric oxide synthase gene with essential hypertension resistant to conventional therapy. *Biochem. Biophys. Res. Commun.* 284:426-430.

107. Wedgwood, S., Mitchell, C.J., Fineman, J.R., and Black, S.M. 2003. Developmental differences in the shear stress-induced expression of endothelial NO synthase: changing role of AP-1. *Am. J. Physiol. Lung Cell Mol. Physiol.* 284:L650-L662.

108. Fleming, I. and Busse, R. 2003. Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 284:R1-12.

109. Wu, G. and Morris, S.M., Jr. 1998. Arginine metabolism: nitric oxide and beyond. *Biochem. J.* 336:1-17.

110. Hattori, Y., Campbell, E.B., and Gross, S.S. 1994. Argininosuccinate synthetase mRNA and activity are induced by immunostimulants in vascular smooth muscle. Role in the regeneration or arginine for nitric oxide synthesis. *J. Biol. Chem.* 269:9405-9408.

111. Hammermann, R., Hirschmann, J., Hey, C., Mossner, J., Folkerts, G., Nijkamp, F.P., Wessler, I., and Racke, K. 1999. Cationic proteins inhibit L-arginine uptake in rat alveolar macrophages and tracheal epithelial cells. Implications for nitric oxide synthesis. *Am. J. Respir. Cell Mol. Biol.* 21:155-162.

112. Nagasaki, A., Gotoh, T., Takeya, M., Yu, Y., Takiguchi, M., Matsuzaki, H., Takatsuki, K., and Mori, M. 1996. Coinduction of nitric oxide synthase, argininosuccinate synthetase, and argininosuccinate lyase in lipopolysaccharide-treated rats. RNA blot, immunoblot, and immunohistochemical analyses. *J. Biol. Chem.* 271:2658-2662.

113. Bellocq, A., Suberville, S., Philippe, C., Bertrand, F., Perez, J., Fouqueray, B., Cherqui, G., and Baud, L. 1998. Low environmental pH is responsible for the induction of nitric-oxide synthase in macrophages. Evidence for involvement of nuclear factor- kappaB activation. *J. Biol. Chem.* 273:5086-5092.

114. Xie, L. and Gross, S.S. 1997. Argininosuccinate synthetase overexpression in vascular smooth muscle cells potentiates immunostimulant-induced NO production. *J. Biol. Chem.* 272:16624-16630.

115. Munder, M., Eichmann, K., Moran, J.M., Centeno, F., Soler, G., and Modolell, M. 1999. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *J. Immunol.* 163:3771-3777.

116. Bronte, V., Serafini, P., Mazzoni, A., Segal, D.M., and Zanovello, P. 2003. L-arginine metabolism in myeloid cells controls T-lymphocyte functions. *Trends Immunol.* 24:302-306.

117. Hirayama, K., Shimoji, M., Swick, L., Meyer, A., and Kapatos, G. 2001. Characterization of GTP cyclohydrolase I gene expression in the human neuroblastoma SKN-BE(2)M17: enhanced transcription in response to cAMP is conferred by the proximal promoter. *J. Neurochem.* 79:576-587.

118. Thony, B., Auerbach, G., and Blau, N. 2000. Tetrahydrobiopterin biosynthesis, regeneration and functions. *Biochem. J.* 347 Pt 1:1-16.

119. Cai, S., Alp, N.J., McDonald, D., Smith, I., Kay, J., Canevari, L., Heales, S., and Channon, K.M. 2002. GTP cyclohydrolase I gene transfer augments intracellular tetrahydrobiopterin in human endothelial cells: effects on nitric

oxide synthase activity, protein levels and dimerisation. *Cardiovasc. Res.* 55:838-849.

120. Saura, M., Perez-Sala, D., Canada, F.J., and Lamas, S. 1996. Role of tetrahydrobiopterin availability in the regulation of nitric- oxide synthase expression in human mesangial cells. *J. Biol. Chem.* 271:14290-14295.

121. Delgado-Esteban, M., Almeida, A., and Medina, J.M. 2002. Tetrahydrobiopterin deficiency increases neuronal vulnerability to hypoxia. *J. Neurochem.* 82:1148-1159.

122. Sakai, N., Kaufman, S., and Milstein, S. 1993. Tetrahydrobiopterin is required for cytokine-induced nitric oxide production in a murine macrophage cell line (RAW 264). *Mol. Pharmacol.* 43:6-10.

123. Hesslinger, C., Kremmer, E., Hultner, L., Ueffing, M., and Ziegler, I. 1998. Phosphorylation of GTP cyclohydrolase I and modulation of its activity in rodent mast cells. GTP cyclohydrolase I hyperphosphorylation is coupled to high affinity IgE receptor signaling and involves protein kinase C. *J. Biol. Chem.* 273:21616-21622.

124. Milstien, S., Jaffe, H., Kowlessur, D., and Bonner, T.I. 1996. Purification and cloning of the GTP cyclohydrolase I feedback regulatory protein, GFRP. *J. Biol. Chem.* 271:19743-19751.

125. Kapatos, G., Hirayama, K., Shimoji, M., and Milstien, S. 1999. GTP cyclohydrolase I feedback regulatory protein is expressed in serotonin neurons and regulates tetrahydrobiopterin biosynthesis. *J. Neurochem.* 72:669-675.

126. Huisman, A., Vos, I., van Faassen, E.E., Joles, J.A., Grone, H.J., Martasek, P., van Zonneveld, A.J., Vanin, A.F., and Rabelink, T.J. 2002. Anti-inflammatory effects of tetrahydrobiopterin on early rejection in renal allografts: modulation of inducible nitric oxide synthase. *FASEB J.* 16:1135-1137.

127. May, J.M. 2000. How does ascorbic acid prevent endothelial dysfunction? *Free Radic. Biol. Med.* 28:1421-1429.

128. Baker, T.A., Milstien, S., and Katusic, Z.S. 2001. Effect of vitamin C on the availability of tetrahydrobiopterin in human endothelial cells. *J. Cardiovasc. Pharmacol.* 37:333-338.

129. Kone, B.C., Kuncewicz, T., Zhang, W., and Yu, Z.Y. 2003. Protein interactions with nitric oxide synthases: controlling the right time, the right place, and the right amount of nitric oxide. *Am. J. Physiol. Renal Physiol.* 285:F178-F190.

130. Liu, J. and Sessa, W.C. 1994. Identification of covalently bound amino-terminal myristic acid in endothelial nitric oxide synthase. *J. Biol. Chem.* 269:11691-11694.

131. Ju, H., Zou, R., Venema, V.J., and Venema, R.C. 1997. Direct interaction of endothelial nitric-oxide synthase and caveolin-1 inhibits synthase activity. *J. Biol. Chem.* 272:18522-18525.

132. Gonzalez, E., Kou, R., Lin, A.J., Golan, D.E., and Michel, T. 2002. Subcellular targeting and agonist-induced site-specific phosphorylation of endothelial nitric-oxide synthase. *J. Biol. Chem.* 277:39554-39560.

133. Bucci, M., Gratton, J.P., Rudic, R.D., Acevedo, L., Roviezzo, F., Cirino, G., and Sessa, W.C. 2000. In vivo delivery of the caveolin-1 scaffolding domain inhibits nitric oxide synthesis and reduces inflammation. *Nat. Med.* 6:1362-1367.

134. Schubert, W., Frank, P.G., Woodman, S.E., Hyogo, H., Cohen, D.E., Chow, C.W., and Lisanti, M.P. 2002. Microvascular hyperpermeability in caveolin-1 (-/-) knock-out mice. Treatment with a specific nitric-oxide synthase inhibitor, L-name, restores normal microvascular permeability in Cav-1 null mice. *J. Biol. Chem.* 277:40091-40098.

135. Venema, V.J., Ju, H., Zou, R., and Venema, R.C. 1997. Interaction of neuronal nitric-oxide synthase with caveolin-3 in skeletal muscle. Identification of a novel caveolin scaffolding/inhibitory domain. *J. Biol. Chem.* 272:28187-28190.

136. Davies, T.H., Ning, Y.M., and Sanchez, E.R. 2002. A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins. *J. Biol. Chem.* 277:4597-4600.

137. Garcia-Cardena, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A., and Sessa, W.C. 1998. Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* 392:821-824.

138. Fontana, J., Fulton, D., Chen, Y., Fairchild, T.A., McCabe, T.J., Fujita, N., Tsuruo, T., and Sessa, W.C. 2002. Domain mapping studies reveal that the M domain of hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release. *Circ. Res.* 90:866-873.

139. Grammatikakis, N., Lin, J.H., Grammatikakis, A., Tsichlis, P.N., and Cochran, B.H. 1999. p50(cdc37) acting in concert with Hsp90 is required for Raf-1 function. *Mol. Cell. Biol.* 19:1661-1672.

140. Dedio, J., Konig, P., Wohlfart, P., Schroeder, C., Kummer, W., and Muller-Esterl, W. 2001. NOSIP, a novel modulator of endothelial nitric oxide synthase activity. *FASEB J.* 15:79-89.

141. Konig, P., Dedio, J., Muller-Esterl, W., and Kummer, W. 2002. Distribution of the novel eNOS-interacting protein NOSIP in the liver, pancreas, and gastrointestinal tract of the rat. *Gastroenterology* 123:314-324.

142. Zimmermann, K., Opitz, N., Dedio, J., Renne, C., Muller-Esterl, W., and Oess, S. 2002. NOSTRIN: a protein modulating nitric oxide release and subcellular

distribution of endothelial nitric oxide synthase. *Proc. Natl. Acad. Sci. U. S. A* 99:17167-17172.

143. Jaffrey, S.R. and Snyder, S.H. 1996. PIN: an associated protein inhibitor of neuronal nitric oxide synthase. *Science* 274:774-777.

144. Chang, Y.W., Jakobi, R., McGinty, A., Foschi, M., Dunn, M.J., and Sorokin, A. 2000. Cyclooxygenase 2 promotes cell survival by stimulation of dynein light chain expression and inhibition of neuronal nitric oxide synthase activity. *Mol. Cell. Biol.* 20:8571-8579.

145. Yvon, A.M., Gross, D.J., and Wadsworth, P. 2001. Antagonistic forces generated by myosin II and cytoplasmic dynein regulate microtubule turnover, movement, and organization in interphase cells. *Proc. Natl. Acad. Sci. U. S. A* 98:8656-8661.

146. Rodriguez-Crespo, I., Yelamos, B., Roncal, F., Albar, J.P., Ortiz de Montellano, P.R., and Gavilanes, F. 2001. Identification of novel cellular proteins that bind to the LC8 dynein light chain using a pepscan technique. *FEBS Lett.* 503:135-141.

147. Hemmens, B., Woschitz, S., Pitters, E., Klosch, B., Volker, C., Schmidt, K., and Mayer, B. 1998. The protein inhibitor of neuronal nitric oxide synthase (PIN): characterization of its action on pure nitric oxide synthases. *FEBS Lett.* 430:397-400.

148. Pan, J., Burgher, K.L., Szczepanik, A.M., and Ringheim, G.E. 1996. Tyrosine phosphorylation of inducible nitric oxide synthase: implications for potential post-translational regulation. *Biochem. J.* 314:889-894.

149. Harris, M.B., Ju, H., Venema, V.J., Liang, H., Zou, R., Michell, B.J., Chen, Z.P., Kemp, B.E., and Venema, R.C. 2001. Reciprocal phosphorylation and regulation of endothelial nitric-oxide synthase in response to bradykinin stimulation. *J. Biol. Chem.* 276:16587-16591.

150. Montagnani, M., Chen, H., Barr, V.A., and Quon, M.J. 2001. Insulin-stimulated activation of eNOS is independent of Ca2+ but requires phosphorylation by Akt at Ser(1179). *J. Biol. Chem.* 276:30392-30398.

151. Fulton, D., Fontana, J., Sowa, G., Gratton, J.P., Lin, M., Li, K.X., Michell, B., Kemp, B.E., Rodman, D., and Sessa, W.C. 2002. Localization of endothelial nitric-oxide synthase phosphorylated on serine 1179 and nitric oxide in Golgi and plasma membrane defines the existence of two pools of active enzyme. *J. Biol. Chem.* 277:4277-4284.

152. Morrow, V.A., Foufelle, F., Connell, J.M., Petrie, J.R., Gould, G.W., and Salt, I.P. 2003. Direct activation of AMP-activated protein kinase stimulates nitric-oxide synthesis in human aortic endothelial cells. *J. Biol. Chem.* 278:31629-31639.

153. Michell, B.J., Chen, Z., Tiganis, T., Stapleton, D., Katsis, F., Power, D.A., Sim, A.T., and Kemp, B.E. 2001. Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. *J. Biol. Chem.* 276:17625-17628.

154. Lin, M.I., Fulton, D., Babbitt, R., Flemming, I., Busse, R., Pritchard, K.A., Jr., and Sessa, W.C. 2003. Phosphorylation of threonine 497 in endothelial nitric oxide synthase coordinates the coupling of L-arginine metabolism to efficient nitric oxide production. *J. Biol. Chem.* 278:44719-44726.

155. Matsubara, M., Hayashi, N., Jing, T., and Titani, K. 2003. Regulation of endothelial nitric oxide synthase by protein kinase C. *J. Biochem. (Tokyo)* 133:773-781.

156. Masini, E., Salvemini, D., Pistelli, A., Mannaioni, P.F., and Vane, J.R. 1991. Rat mast cells synthesize a nitric oxide like-factor which modulates the release of histamine. *Agents Actions* 33:61-63.

157. Kanwar, S., Wallace, J.L., Befus, D., and Kubes, P. 1994. Nitric oxide synthesis inhibition increases epithelial permeability via mast cells. *Am. J. Physiol.* 266:G222-G229.

158. Koranteng, R.D., Dearman, R.J., Kimber, I., and Coleman, J.W. 2000. Phenotypic variation in mast cell responsiveness to the inhibitory action of nitric oxide. *Inflamm. Res.* 49:240-246.

159. Heywood, G.J. and Thomas, P.S. 2002. Nicorandil inhibits degranulation and TNF-alpha release from RBL-2H3 cells. *Inflamm. Res.* 51:176-181.

160. Messina, A., Knight, K.R., Dowsing, B.J., Zhang, B., Phan, L.H., Hurley, J.V., Morrison, W.A., and Stewart, A.G. 2000. Localization of inducible nitric oxide synthase to mast cells during ischemia/reperfusion injury of skeletal muscle. *Lab. Invest.* 80:423-431.

161. Forsythe, P. and Befus, A.D. 2003. Inhibition of calpain is a component of nitric oxide-induced down-regulation of human mast cell adhesion. *J. Immunol.* 170:287-293.

162. Apgar, J.R. 1997. Increased degranulation and phospholipase A2, C, and D activity in RBL cells stimulated through FcepsilonR1 is due to spreading and not simply adhesion. *J. Cell Sci.* 110 (Pt 6):771-780.

163. Clements, M.K., Siemsen, D.W., Swain, S.D., Hanson, A.J., Nelson-Overton, L.K., Rohn, T.T., and Quinn, M.T. 2003. Inhibition of actin polymerization by peroxynitrite modulates neutrophil functional responses. *J. Leukoc. Biol.* 73:344-355.

164. Forsythe, P., Gilchrist, M., Kulka, M., and Befus, A.D. 2001. Mast cells and nitric oxide: control of production, mechanisms of response. *Int. Immunopharmacol.* 1:1525-1541.

165. Coleman, J.W. 2002. Nitric oxide: a regulator of mast cell activation and mast cell- mediated inflammation. *Clin. Exp. Immunol.* 129:4-10.

Chapter 2.

Nitric Oxide Synthase and Nitric Oxide Production in In-Vivo Derived Mast Cells

Authors, M.Gilchrist, M.Savoie, O. Nohara, F.L. Wills, J.L. Wallace and A.D. Befus. A version of this chapter was published in Journal of Leukocyte Biology. 2002. I contributed to this work by designing and carrying out the experiments and writing the manuscript.

Introduction

Nitric Oxide (NO) is a remarkable molecule, functioning as both a cellular messenger and cytotoxic agent. This short-lived mediator is formed by the sequential oxidation of the substrate L-arginine by the nitric oxide synthase (NOS) family of enzymes, with the formation of L-citrulline and NO as the byproduct (1). NO has been implicated in numerous homeostatic functions including: vasodilation (2), neurotransmission (3), and host defense against pathogens (4), but also in pathological conditions such as sepsis, autoimmune diseases, asthma and other inflammatory diseases (5-7). In the latter conditions, excessive production of NO by the Ca^{2+} independent inducible isoform iNOS is considered to be important (8).

Mast cells (MC) are strategically located throughout the body at neural, epidermal, mucosal, and vascular sites. They play a critical role in allergy and other inflammatory states through the release of multifunctional mediators such as histamine, cytokines, arachidonic acid metabolites and proteinases (9). Production of NOS and NO by rat MC has been previously investigated by a variety of means, in many MC types, and using a variety of time courses. Direct localization of iNOS protein to MC has been described using immunohistochemistry in rat intestinal biopsies, mouse cultured MC and rat brain tissue (10-12). Bioassays, including platelet aggregation (13,14) and epithelial permeability (15) have been used to indirectly detect the production of NO by MC after short-term activation in both mouse and rat PMC. Detection of iNOS mRNA was accomplished in MC cultured from mouse bone marrow (11) and subsequently stimulated with IgE. The NO produced by mouse MC has been detected and quantified as its stable breakdown product nitrite (NO_2^-) using the Griess assay following long term stimulation with IgE (11). However, the amounts of NO obtained were low and there was little information concerning events at the cellular level. Furthermore, there is debate about whether NO is made by the MC or by contaminating cells in isolated cell populations (14), or if indeed MC have the cellular capacity to react to cytokines like IFN-γ to produce NO (16). Therefore, despite recent studies giving further indication that rodent MC produce NO (11,12), the NOS isoform involved, the balance

between constitutive and inducible NO production, and the precise stimulatory events and time-course that initiate the NO production remain unresolved.

To investigate the regulation of NO in PMC, we studied the expression of NOS isoforms constitutively and following induction by cytokines and antigen. In this study we demonstrate the production of constitutive eNOS mRNA and upregulated iNOS mRNA expression by PMC after treatment with IFN-γ, stimulating antigen or CD8 ligation with antibody. Secondly, we localize the iNOS mRNA directly to MC by an *in-situ* RT-PCR technique. Thirdly we demonstrate that PMC synthesize iNOS protein as detected by immunohistochemistry. Lastly, this protein is active and stimulates the production of NO.

Materials and Methods

Animals

Adult male Sprague-Dawley (Crl:CD (SD) BR) rats were obtained from Charles River Canada Inc. (Quebec, Canada) and maintained in an isolated room in filter-top cages to minimize unwanted infections. For experiments in which PMC were to be isolated, then stimulated with antigen, rats were sensitized by infection with L_3 larvae of *Nippostrongylus brasiliensis* 5 to 6 weeks before MC isolation as previously described (17). Food and water were provided *ad libitum* and animals were maintained on a 12h light-12h dark (0700 to 1900) cycle. Experimental procedures were approved by the University Animal Care Committee and were in accordance with the guidelines of the Canadian Council on Animal Care.

Antigen

Antigen (Ag) used to activate in vivo sensitized PMC was a collection of soluble excretory and secretory products of adult *N. brasiliensis* prepared by incubating worms in phosphate buffered solution (pH 7.2) at 37°C for 4 h (18). The antigen concentration was described as worm equivalents (WE) per mL, and the final protein concentration of Ag (5 WE/mL) was 7.1 ± 0.8 μg/mL. LPS levels were negative as tested by E-toxate reagent (SIGMA).

MC isolation and stimulation

Total peritoneal cells were obtained by peritoneal lavage with 15 mL of cold HEPES (10mM)-buffered Tyrode's solution containing 0.1% BSA. PMC were purified by centrifuging the total peritoneal cells through a two-step discontinuous gradient of Percoll (Pharmacia Ltd., Uppsala, Sweden) as previously described (18). The number of PMC recovered was 1.1 x 10^6 cells/rat, and PMC purity was >98% as determined by staining with toluidine blue. Contaminating cells (<2%) included eosinophils, pycnotic neutrophils, small dense lymphocytes and erythrocytes. Cell viability, as determined by trypan blue staining was >99%. PMC were resuspended in RPMI-1640 medium (Gibco/BRL, Grand Island, NY) containing L-glutamine pH 7.2, supplemented with 5% heat inactivated FBS, and 40U/mL penicillin/streptomycin (GIBCO/BRL). For RT-PCR experiments PMC from unsensitized rats were treated with medium containing either IFN-γ (800 U/mL)(Gibco/BRL) or anti-CD8 antibody (OX-8, 5 μg/mL)(Serotec, Toronto, Canada) for 6 h. PMC from sensitized rats were treated with sensitizing antigen (5 WE/mL) for 16 h. As controls for all experiments, PMC from unstimulated rats were incubated without treatment for the described period of time. For immunohistochemistry experiments, PMC were stimulated with IFN-γ (800 U/mL), anti-CD8 (5 μg/mL) or antigen (5 WE/mL) for 16h. As a positive control, isolated macrophages were also incubated with IFN-γ (800 U/mL)(Gibco/BRL) for 16 h. For nitrite (NO_2^-) determinations PMC were incubated with IFN-γ (800 U/mL), anti-CD8 (5 μg/mL), or antigen (5 WE/mL) for 48 h.

Reverse transcription (RT)-PCR

Total RNA was isolated from PMC using a modification of the Chomczynski/Sacchi method as previously described. The isolated RNA was then pretreated with heparinase I (Sigma Chemical Co., St. Louis, MO) to remove contaminating heparin (19). One μg of RNA was converted to cDNA by the reverse transcription reaction (M.MLv reverse transcriptase, GIBCO/BRL) in a total volume of 20 μL. PCR amplification was performed in a final volume of 20 μL to which 2 μL of cDNA had been added. The reaction was performed on a PTC-100 Thermal Cycler (MJ Research, Boston, MA). The primers were designed to be intron-spanning and the expected product sizes are

listed in Table 2.I. The number of cycles used for the NOS primer sets was 25-30, (depending on the linear range) and 20 cycles for β–actin. The conditions for PCR amplification are as follows: denaturing at 95°C for 45 s, annealing for 45s, and extension at 72°C for 2 min. The annealing temperature was 56°C for both β-actin and iNOS, 54°C for eNOS and 50°C for nNOS. For semi-quantitative PCR analysis of iNOS induction, during amplification aliquots were withdrawn at 26, 29, 32 and 35 cycles to insure that amplification was within the exponential range. Products were run on a 1.2 % agarose gel and stained with ethidium bromide (Sigma).

In situ RT-PCR

In-situ RT-PCR was performed as previously described (20,21). Briefly, PMC were washed in DEPC-treated PBS then fixed for 16 h in 10% buffered formalin, washed twice with DEPC-treated water, then placed on slides and allowed to dry overnight. The slides were immersed in 2mg/mL (7000 U/mL) pepsin (Boehringer Mannheim, Mannheim, Germany) in 0.01N HCl for 40 min at room temperature, then treated with RNase-free DNase I (Boehringer Mannheim) at 37 °C overnight. The test specimens were treated with a reverse transcription solution containing the downstream primer (1 μM) and MMLV-RT as the enzyme. Amplification of the cDNA was accomplished with a PCR solution containing 4.5mM $MgCl_2$; 200 μM each of dATP, dCTP, dGTP, and dTTP; 1 μM of each primer; 10 μM digoxigenin-11-dUTP (Boehringer Mannheim), 0.8 μM of iNOS primers (same set as for solution-phase RT-PCR) and 5 U of Taq polymerase (GIBCO/BRL). Cycling conditions were 5 min at 94 °C, and 30 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1.5 min. The digoxigenin-11-dUTP labeled PCR product was detected after incubation with alkaline phosphatase anti-digoxigenin conjugate (Boehringer Mannheim) (1/200 dilution in 0.1 M Tris-HCl, 0.1 M NaCl , 2 mM $MgCl_2$ pH 7.2) for 30 min at room temperature, and development in NBT/BCIP (Boehringer Mannheim) substrate solution for 30 min. Control spots, both positive and negative, were run concurrently on each test slide. Positive control spots without DNase treatment were used to optimize the length of pepsin digestion. When digestion and PCR conditions were optimal there was strong nuclear amplification. Negative controls, where the cells were treated with DNase and the RT

Table 2.I Primer sets used for reverse-transcriptase polymerase chain reaction experiments.

RNA detected	Primer pair (sense; anti-sense)	Product size (bp)
β-actin	GTGGGGCGCCCCAGGCACCA; GTCCTTAATGTCACGCACGATTTC	526
nNOS	CCTTGGTAGACCTCAGCTATGA; TTGCCATCGAGGTCTCTGTCCA	513
iNOS	ACAACAGGAACCTACCAGCTCA; GATGTTGTAGCGCTGTGTGTCA	651
eNOS	GGAGAAGATGCCAAGGCTGCTG; CTTCCAGTGTCCAGACGCACCA	224

step eliminated, showed that no priming by genomic DNA was detectable and that detected products in the test spot were the result of amplified mRNA.

Immunohistochemistry for iNOS

Immunohistochemistry was performed on preparations of PMC to further localize iNOS protein within the cells and to confirm that the iNOS detected was derived from PMC and not from contaminating cells. Briefly, IFN-γ, anti-CD8, or antigen-treated PMC along with untreated PMC were washed in PBS and then cytocentrifuged onto slides and air-dried overnight. After treatment with 3% hydrogen peroxide to block endogenous peroxidase, slides were incubated overnight at 4°C with a 1/100 (1 μg/mL) dilution of monoclonal iNOS antibody (Santa Cruz, Santa Cruz CA). Specific antibody binding was detected with a peroxidase-labeled avidin-biotin complex (Vector Laboratories), with diaminobenzidine used as the chromagen. Negative control cytospins labeled with isotype (IgG1) antibody control at a concentration of 1ug/mL were run concurrently. To confirm the specificity of staining, iNOS antibody was adsorbed with immunizing peptide according to the manufacturer's protocol (Santa Cruz), and this adsorbed antibody was also used concurrently to stain PMC.

Measurement of NO_2^- production

NO_2^- in culture (phenol red free) supernatants was measured by the Griess reaction (22). Results were expressed as μM/2 $x10^5$ cells/48 h following incubation of $2x10^5$ cell in 200 μL volume. Equal volumes of cell free supernatant and Griess reagent (1% sulfanilamine, 0.1% N-(1-naphyl)-ethylene-diamine dihydrochloride, 2.5% H_3PO_4) were mixed. $NaNO_2$ was used as a standard. Plates were read on a Vmax kinetic microplate reader (Molecular Devices Co., Menlo Park, CA) at 540 nm.

Assay of NOS Activity

To further categorize NO production in PMC, NOS activity was measured by the conversion of L-[^{14}C] arginine to L-[^{14}C] citrulline, as described previously (23). PMC (5×10^6) were incubated in the presence of 800 U/mL IFN-γ for 18 h, unstimulated PMC (control) were isolated and used immediately (0 h). The cells were centrifuged at 900 x g for 5min and the resulting pellet was retained. The pellet was homogenized in buffer containing 50 mM Tris HCl, 320 mM sucrose, 1 mM dithiothreitol, 10 μg/mL

leupeptin, 10 μg/mL soybean trypsin inhibitor, and 2 μg/mL aprotinin (Sigma). To start the assay, 40 μL of cell homogenate was added to tubes containing 100 μL of reaction buffer consisting of 50 mM KH_2PO_4 (pH 7.2), 50 mM valine, 100 μM NADPH, 1 mM L-citrulline, 0.2 mM $CaCl_2$, 5 μM tetrahydrobiopterin, 20 μM L-arginine and L-[^{14}C]arginine (ICN). Duplicate incubations at 37 °C for each sample were run for 30 min in the presence of EGTA (2mM) to determine the levels of calcium-dependent (constitutive) NOS activity. Calcium-independent (inducible) NOS activity was determined by subtracting the constitutive activity from the total NOS activity in the sample. The reaction was terminated by the addition of 1.0 mL of Dowex resin AF 50W-X8 (pH 7.5)(Sigma). The resin was allowed to settle for 45 min, then 500 μL of the supernatant was removed and analyzed by liquid scintillation counting. The level of citrulline produced was expressed as picomoles/min/mg of protein. The protein content was determined by the Bradford technique (BioRad) using (BSA) as a standard. The Citrulline assay was validated by determining the retention of radioactive arginine by the Dowex resin. 100 μL of reaction cocktail were applied to 1.0 mL of Dowex resin and counts determined as above. Greater than 99% of the applied radioactivity was retained by the Dowex resin. Blank values obtained from sample tubes containing boiled cell extracts were subtracted from all test samples. Specificity of the assay was further validated by adding the pan-NOS inhibitor, NG -monomethyl- L-arginine (L-NMMA) (100 μM), to ensure that citrulline detected arose from the activity of NOS.

Results

Analysis of NOS expression in unstimulated PMC

As PMC are known to spontaneously produce NO (24), we examined the expression of NOS enzymes in unstimulated PMC by RT-PCR. Specific primers for nNOS, iNOS and eNOS were designed from genbank sequences (Table 2.I). PMC express detectable eNOS mRNA after 30 cycles. In our hands, unstimulated PMC produce no detectable nNOS or iNOS mRNA (Fig. 2.1). In all reactions the validity of the PCR was indicated by the presence of products of the suitable size with the appropriate positive control, rat brain (nNOS), IFN-γ stimulated macrophages (iNOS) and rat kidney (eNOS),

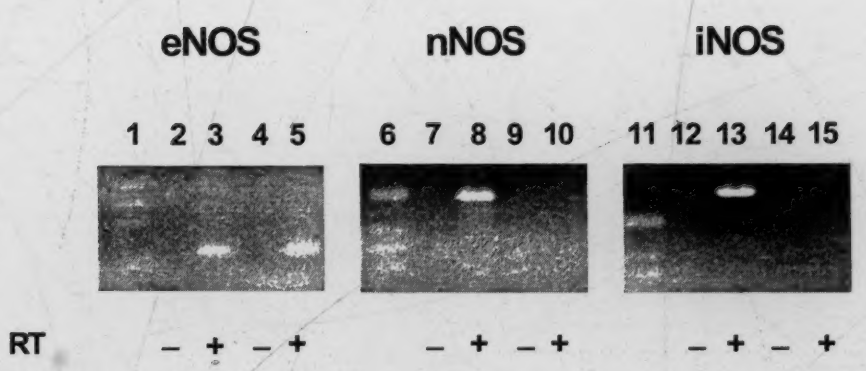


Fig. 2.1. Expression of nitric oxide synthase (NOS) messenger RNA in unstimulated rat peritoneal mast cells (PMC). Representative RT-PCR analysis of total RNA isolated from untreated PMC using endothelial NOS (eNOS)(lanes 2-5), neuronal NOS (nNOS) (lanes 7-10) and inducible NOS (iNOS)(lanes 12-15) specific primers. RT negative controls are as indicated. Rat kidney (lanes 2 and 3), rat brain (lanes 7 and 8), and interferon-γ (IFN-γ) stimulated rat macrophages (lanes 12 and 13) were used as the appropriate controls. Molecular weight markers are in lanes 1, 6 and 11. Results are representative of four different RNA isolations.

respectively. All PCR reactions were negative when the RT step was eliminated, indicating that there was no contamination from genomic DNA.

Analysis of iNOS mRNA expression by RT-PCR

Total RNA was extracted from unstimulated PMC (>98% pure), and from PMC treated *in-vitro* with optimized concentrations of known inducers of iNOS expression (IFN-γ, CD8 ligation, IgE cross-linking with stimulating antigen). iNOS mRNA production was then assessed by semiquantitative RT-PCR (Fig. 2.2). iNOS signal (26 cycles) in PMC increased following treatment with all three stimulants. IFN-γ (0.71±0.05 arbitrary units (a.u.)) or anti-CD8 (0.70±0.08 a.u.) treatment produced approximately twofold greater iNOS mRNA induction compared to stimulating antigen (0.37±0.12 a.u.) as measured by band densitometry verses a β-actin control. eNOS mRNA remained unaltered when PCR was run on the same samples. As seen previously, no nNOS mRNA was detected under any of the treatment conditions studied. Consistent with our initial studies on unstimulated PMC, iNOS expression was not detected in untreated PMC. Results are from PMC RNA obtained from three independent batches of cells.

iNOS mRNA localization by in situ RT-PCR

The above experiments do not rule out the possibility that iNOS production in rat PMC could be attributed to other contaminating peritoneal cells, such as macrophages and neutrophils (16). Accordingly, *in situ* RT-PCR was employed to localize the iNOS mRNA and to confirm that MC are the source of the products detected in the RT-PCR experiments. Both untreated and anti-CD8 treated cells were fixed and analyzed. Cells in the positive control spots (cells treated with pepsin, but with no DNase and no RT step) of each slide showed intense nuclear staining indicating the successful synthesis and detection of amplified genomic DNA. This result indicates that pepsin digestion and PCR conditions are optimal. Cells in the negative control spots (cells treated with pepsin and DNase, but with no RT step) showed no signal in any cellular compartment. This result indicates that signal seen in test spots do not arise from genomic DNA. Cells on test spots (cells treated with pepsin, DNase, and an RT step) were scored as positive if strong staining was localized within the cytoplasm alone; 200 cells per slide were

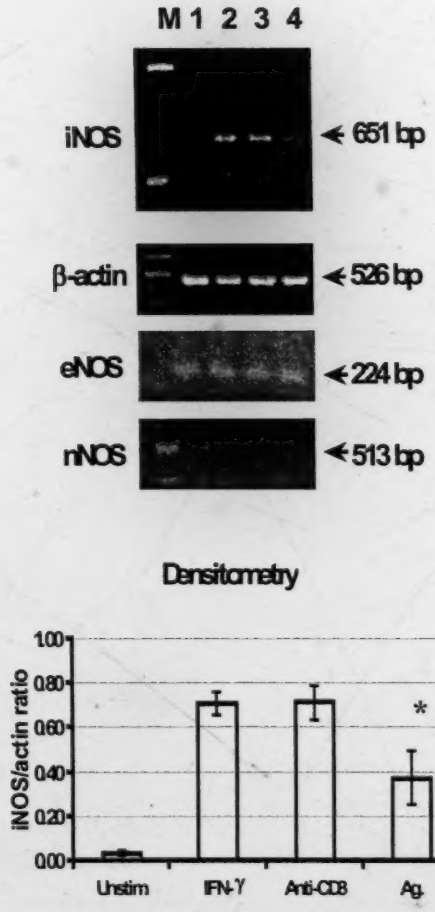


Fig. 2.2. Measurement of messenger RNA (mRNA) levels for inducible nitric oxide synthase (iNOS) and β–actin by RT-PCR in rat peritoneal mast cells (PMC) cultured in media alone (1), with 800U/mL interferon-γ (IFN-γ) (2), with anti-CD8 (5μg/mL) (3) and with antigen (Ag) (5 we/mL) for 6 h. Densitometric analyses are shown as the iNOS/β-actin ratio, expressed in arbitrary units. Endothelial NOS (eNOS) mRNA levels are stable under similar treatment conditions. No neuronal NOS (nNOS) mRNA was detected. The PCR gels shown are representative of two others performed using PMC from different cell isolations. Error bars represent SEM from three separate experiments. *, Indicates $p < 0.05$ by comparison with IFN-γ or anti-CD8 treated cells.

analyzed, using three independent batches of PMC. The anti-CD8 treated PMC showed significant ($p<0.001$) induction of iNOS in 70.5±2.8 % of the cells. In the untreated cell population 13.7±1.5 % of PMC show localization of iNOS mRNA to the perinuclear region. (Fig. 2.3). The localization of mRNA for iNOS in all PMC was in the perinuclear region, as previously described for other mRNA in these cells (21). In general there was an increase in the number of cells positive, rather than an overall increase in intensity or distribution within cells themselves. These results confirm that PMC produce mRNA for iNOS.

Immunohistochemistry

To confirm the cellular source of iNOS protein, immunohistochemical analysis was performed. Staining for iNOS protein in stimulated PMC was detected with all treatments (IFN-γ shown)(Fig. 2.4b). Cell counts revealed that upon IFN-γ treatment, 97.5± 0.4 % of cells were positive. iNOS immunostaining was also increased in anti-CD8 and antigen stimulated cells, 96.2 ± 0.6 % and 94.1 ± 0.3 % respectively. Staining was predominantly in the cytoplasm (arrows), with some protein also being localized to the granules (arrowheads). Very little iNOS staining (4.3 ± 0.7 %) was detected in untreated PMC (Fig. 2.4a). This data is summarized in Table 2.II. Staining was blocked when the iNOS antibody was preincubated with the iNOS immunizing peptide (Fig. 2.4c), staining was also absent in slides incubated with IgG1 control antisera (Fig. 2.4d).

NO production in PMC

To determine if PMC produce functional iNOS protein, cells treated with IFN-γ, anti-CD8, or antigen were compared to untreated PMC for their NO production capacity. Upon activation with IFN-γ, anti-CD8, or antigen (all present for 48 h), PMC released significantly greater ($p<0.01$) amounts of NO_2^- (7.6±0.9, 6.6±0.9, and 8.4±0.6 μM/2 x 10^5/48 h, respectively). Unstimulated PMC spontaneously released low levels of NO_2^- after 48 h of incubation (2.1±0.3 μM/2 x 10^5/48 h) (Fig. 2.5). To determine if the NO produced arises from the conversion of L-arginine by NOS, the NOS inhibitor, (L-NMMA) (100 μM), was added concomitantly to IFN-γ stimulated cells. NO production was reduced back to below baseline levels in the presence of L-NMMA.

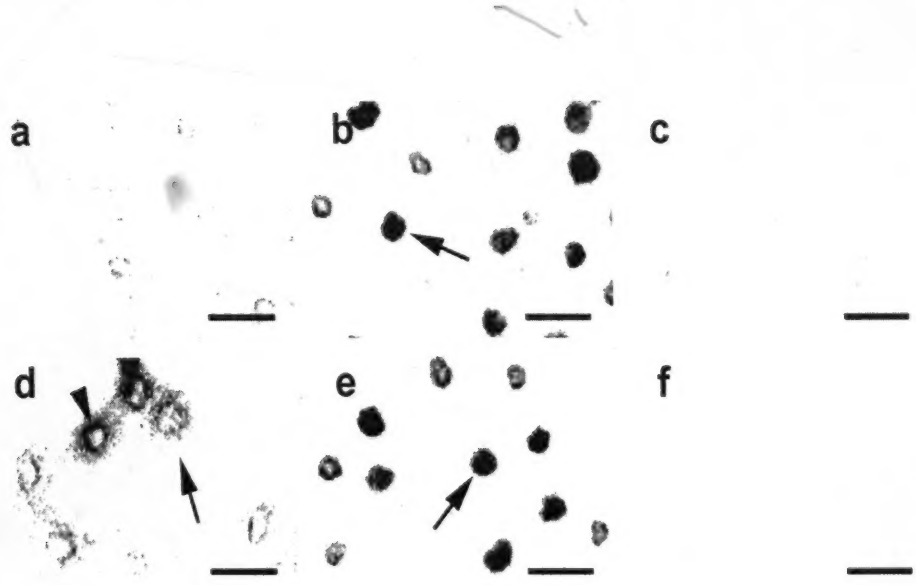


Fig. 2.3. *in-situ* reverse-transcriptase polymerase chain reaction (RT-PCR) localization of inducible nitric oxide synthase (iNOS) messenger RNA (mRNA) in formalin fixed smears of untreated rat peritoneal mast cells (PMC) (*a, b,* & *c.*) and anti-CD8 (5 μg/mL) stimulated PMC (*d, e,* & *f*). In *a*, only a few untreated cells show positivity for iNOS mRNA (13.7±1.5%), whereas in *d* a significant ($p<0.001$) percentage (70.5±2.8%) of anti-CD8 stimulated cells are positive (arrow). Note the intense perinuclear signal (arrowhead). In *b* & *e*, all cells showed intense nuclear staining (arrow) in the positive control indicating nuclear DNA priming and that the pepsin treatment was optimal, thereby allowing PCR reagents to enter the cell. The cytoplasm remains negative. In *c* and *f*, the cells were treated with DNase and the RT step was eliminated, no staining was shown in the negative control, indicating that the product detected was indeed cDNA and not from contaminating genomic DNA. Original magnification: X 1,000, bar = 10 μm. Results are representative of three separate experiments, counting 200 cells per slide.

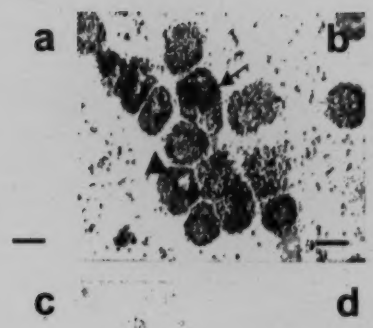


Fig. 2.4. Immunohistochemical identification of inducible nitric oxide synthase (iNOS) protein in rat peritoneal mast cells (PMC). Staining for iNOS protein in interferon-γ (IFN-γ) stimulated PMC was detected (Fig. 4b) predominantly in the cytoplasm (arrows), with some protein also being localized to the granules (arrowheads). Few iNOS staining cells were detected in untreated PMC (Fig. 4a). Staining was blocked when the iNOS antibody was preincubated with the iNOS immunizing peptide (Fig. 4c), staining was also absent in slides incubated with IgG1 control antisera (Fig. 4d). Original magnification: x 1,000, bar = 10 μm.

Table 2.II Immunohistochemical analysis of inducible nitric oxide synthase expression in rat peritoneal mast cells.

Cells	% positive cells	# of experiments
Unstimulated PMC	4.3 ± 0.7	3
PMC + IFN-γ	97.5 ± 0.4	3
PMC + Anti-CD8	Range : 95.0-98.0	2
PMC + Antigen	Range : 93.0-96.0	2

Isolated peritoneal mast cell (PMC) were incubated in control medium or in medium containing either interferon-γ (IFN–γ) (800 U/mL), anti-CD8 (5 μg/mL) or antigen (5 WE/mL) for 16 h at 37°C. Cells were spun onto slides and inducible nitric oxide synthase (iNOS) protein identified by immunohistochemistry. Controls include staining with IgG1 control sera, as well as iNOS antisera incubated with the immunizing peptide. Percentage PMC positive for iNOS using an anti-iNOS antibody, based on scoring 200 cells per independent experiments ± SD.

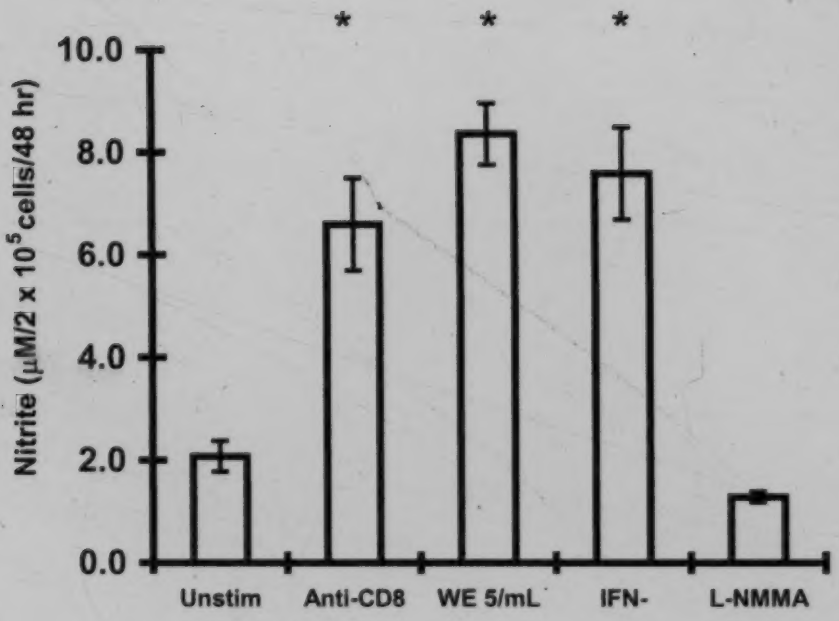


Fig. 2.5. The release of nitric oxide (NO) (as measured by nitrite) by the Griess assay from rat peritoneal mast cells (PMC) stimulated with anti-CD8, antigen, and interferon-γ (IFN-γ). Cells treated concomitantly with IFN-γ and the NOS inhibitor L-NMMA (100μM) were used to determine the specificity of NO production. Cells from unsensitized rats were used as the unstimulated control. Data shown as mean ± SEM of 5 experiments, 2 x 10^5 cells/sample were analyzed. *, Indicates $p < 0.05$ by comparison with untreated cells.

NOS activity

To confirm that NOS protein is functional, and to assess whether stimulation of NOS activity after IFN-γ treatment was due to the induction of iNOS or to eNOS, the citrulline assay was performed. This assay is based on the conversion of L-arginine to L-citrulline, and allows detection and differentiation of both constitutive and inducible isoenzyme activity in PMC homogenates (Fig. 2.6). Blank values obtained from concordant incubations with heat-treated samples (to inactivate NOS activity) were subtracted from all test values. NOS activity in unstimulated PMC (0 h) was produced exclusively by Ca^{2+}-dependent NOS activity (43.7 ±5.1 pmol/min/mg protein), with no detectable iNOS activity. Treatment with IFN-γ (18 h) significantly ($p < 0.01$) increased citrulline production in PMC by iNOS activity (52.8 ±18.4 pmol/min/mg protein), but with no significant increase in cNOS (eNOS/nNOS) activity (31.8±22.5 pmol/min/mg protein). This correlates with mRNA data showing induction of iNOS message following stimulation with IFN-γ. NOS activity was inhibited by the addition of L-NMMA, indicating that the citrulline conversion detected was due to NOS (data not shown).

Discussion

NO is a biological messenger with diverse and ubiquitous function. It is produced in a variety of cells by the NOS family of enzymes, consisting of both constitutively active (eNOS and nNOS) and inducible (iNOS) members. cNOS produced NO is known for its role as both a signaling molecule within cells, including neurons (3), as well as its role in maintaining vascular tone through modulation of endothelial cGMP levels (2). Conversely, iNOS produced NO is sustained and this can allow interaction with a diverse array of intra- and extracellular targets. iNOS produced NO modulates cellular cGMP levels, and is thought to be the reason for the excessive vasodilation seen in septic shock (5). Furthermore, iNOS produced NO also has a varied and complex series of interactions that are cGMP-independent and are reliant on the cellular microenvironment, and as such can be involved in such cellular mechanisms as apoptosis, cytostasis, and destruction of cellular pathogens (5,25).

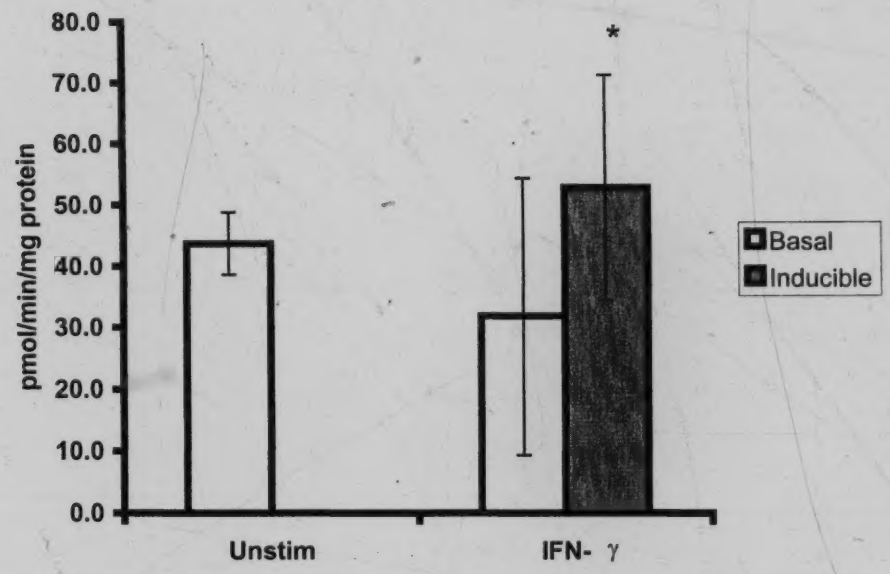


Fig. 2.6. Nitric oxide synthase (NOS) activity in untreated (0 h) and interferon-γ (IFN-γ) (18 h) stimulated rat peritoneal mast cells (PMC), as measured by the citrulline assay. The results are expressed as picomoles L-citrulline formed per minute, per mg of protein. Data shown as mean ± SEM for three independent experiments. *, Indicates $p < 0.05$ by comparison with levels of inducible NOS activity in untreated cells.

We have demonstrated that rat PMC are a source of eNOS and iNOS mRNA as well as iNOS protein. We show that unstimulated PMC express no detectable iNOS or nNOS mRNA. However, low baseline levels of eNOS mRNA are expressed. Interestingly, unstimulated PMC have Ca^{2+}-dependent NOS activity as measured by citrulline production. To our knowledge, this is the first demonstration of Ca^{2+}-dependent NOS activity in PMC. Furthermore, iNOS mRNA is rapidly induced (6 h) upon stimulation with either antigen, anti-CD8 antibody or IFN-γ. This mRNA is also translated, and iNOS protein was detected in stimulated PMC using immunohistochemistry. Furthermore, the translated iNOS protein is biologically active as quantified by the citrulline assay and produces increased levels of NO, measured as nitrite, by the Griess assay.

Since PMC were isolated from mixed peritoneal cells the possibility exists that the NOS/NO expression observed may be due to the presence of contaminating cells such as macrophages. Furthermore, several early reports of MC NO production were derived from populations that contained up to 15% contaminating cells (14), and recent studies have confirmed that in peritoneal cell populations these contaminating cells contributed significantly to the NO produced (16). To confirm the cellular source of iNOS we used *in-situ* RT-PCR to localize the mRNA directly to PMC. PMC treated with anti-CD8 showed a significant increase in the cytoplasmic accumulation of iNOS mRNA after 18 h. As further verification, iNOS protein was confirmed to arise from PMC by the immunocytochemistry results. Interestingly, the pattern of iNOS staining obtained showed similar cytoplasmic and granular localization as that recently described by Messina (12), who showed iNOS immunoreactivity in brain MC after ischemia, providing further evidence that MC produce iNOS protein.

These results present an important point because they differ from the observations of other groups that MC isolated from mixed peritoneal populations do not produce detectable amounts of NO, particularly following IFN-γ stimulation (16,26). This result may be due to methodological differences in the Griess assay, including such variables as the ratio of sample:Griess reagent added (3:1 vs. 1:1), or time of incubation before supernatant removal (24 vs. 48 h). Our results obtained using the more sensitive

citrulline assay, which allows detection of the low amounts of NOS activity found in some cell systems (23), confirms that PMC have low level NOS activity. Unfortunately, neither assay employed allows for the direct cellular detection of NO, thus we are unable to equivocally determine the cellular source.

The fact that PMC produce so little NO in relation to other peritoneal cells, especially macrophages, leads to the question of physiological relevance of PMC derived NO. Indeed Eastmond et al have recently shown that in peritoneal populations, PMC function is regulated substantially by macrophage derived NO (26). However, NO produced by MC in other tissue environments such as in lung or the intestine may make a significant contribution to tissue function. Studies by Gaboury et al show that MC derived NO is important in down regulating neutrophil adhesion to the vascular endothelium (15). Furthermore, studies by Bidri et al (11) on pure MC populations implicated MC in NO production, and investigations using MC lines further show that MC derived NO is important in endogenous regulation of the cell (27). Thus, the contribution of the extracellular environment must be considered when determining the amount and functional significance of MC derived NO.

Since MC have the ability to act in both homeostatic and pathological roles, a precise function of MC derived NO is difficult to delineate. MC NO produced basally through the activity of eNOS could function in a physiological capacity, functioning in signal transduction via cGMP-mediated mechanisms. PMC have previously been shown to spontaneously produce NO without prior stimulation (13,14,24). Since iNOS mRNA is not increased until 4-6 h following activation, these results must be due to the presence of a basally active NOS enzyme. The finding in this study of the presence of a functioning eNOS system can now provide an explanation for the release of basal NO from rat PMC.

By virtue of their ability to produce NO through iNOS upon exposure to various immunological stimuli, MC may play an important role in innate immunity. MC are strategically located throughout the body and have been shown to bind various bacteria often in the absence of opsonization (28). Through the production of NO, MC may function directly or in combination with other mediators and reactive intermediates to

eliminate bacteria and parasites (29). Furthermore, NO produced locally by MC may contribute to general vasodilation and the associated accumulation of inflammatory cells to site of infection (15). Interestingly while crosslinking either IgE receptors or stimulation through CD8 can be considered as possible pro-inflammatory signals, IFN-γ is a well know inhibitor of MC function (26,30,31). It is interesting to postulate that MC NO derived from IFN-activation may mediate MC downregulation. However, recent studies by Deschoolmeester and others have raised the possibility that PMC may not produce function IFN-γ receptors on their cell surface (16). This could be a reason for the requirement for higher concentrations of IFN-γ to stimulate MC to produce NO as compared to that needed to activate macrophages. Whether MC respond specifically to IFN-γ by a receptor or by a non-specific mechanism such as charge interaction, is an area that clearly requires more study, and will rely on confirming the presence of IFN-γ receptor mRNA and protein in MC.

In summary, we have presented evidence for the differential production of NOS isoenzyme mRNA, protein and NO production in unstimulated and treated PMC. However, further definition of the regulatory pathways for NO synthesis will help identify the inflammatory responses favorable to the production of NO by MC and the roles MC NO may play in tissue homeostasis, inflammation and host defense.

References:

1. Nathan, C. 1992. Nitric oxide as a secretory product of mammalian cells. *FASEB. J.* 6:3051-3064.
2. Palmer, R.M., Ferrige, A.G., and Moncada, S. 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327:524-526.
3. Bredt, D.S. and Snyder, S.H. 1992. Nitric oxide, a novel neuronal messenger. *Neuron* 8:3-11.
4. Nathan, C.F. and Hibbs, J.B. 1991. Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr. Opin. Immunol.* 3:65-70.
5. Christopherson, K.S. and Bredt, D.S. 1997. Nitric oxide in excitable tissues: physiological roles and disease. *J. Clin. Invest.* 100:2424-2429.
6. Yeadon, M. and Price, R. 1995. Induction of calcium-independent nitric oxide synthase by allergen challenge in sensitized rat lung in vivo. *Br. J. Pharmacol.* 116:2545-2546.
7. Curran, A.D. 1996. The role of nitric oxide in the development of asthma. *Int. Arch. Allergy Immunol.* 111:1-4.
8. Moncada, S. and Higgs, A. 1993. The L-arginine-nitric oxide pathway. *N. Engl. J. Med.* 329:2002-2012.
9. MacDonald, A.J., Wills, F.L., Lin, T.J. and Befus, A.D. 1996. The role of mast cells in inflammation and homeostasis. In *Allergy and Allergic Diseases: The New Mechanisms and Therapeutics*. (J. Denburg, ed.), The Humana Press, Totowa, NJ. 103-125.
10. Bandaletova, T., Brouet, I., Bartsch, H., Sugimura, T., Esumi, H. and Ohshima, H. 1993. Immunohistochemical localization of an inducible form of nitric oxide synthase in various organs of rats treated with Propionibacterium acnes and lipopolysaccharide. *APMIS.* 101:330-336.
11. Bidri, M., Ktorza, S., Vouldoukis, I., Le Goff, L., Debre, P., Guillosson, J.J. and Arock, M. 1997. Nitric oxide pathway is induced by Fc epsilon RI and up-regulated by stem cell factor in mouse mast cells. *Eur. J. Immunol.* 27:2907-2913.

12. Messina, A., Knight, K.R., Dowsing, B.J., Zhang, B., Phan, L.H., Hurley, J.V., Morrison, W.A. and Stewart, A.G. 2001. Localization of inducible nitric oxide synthase to mast cells during ischemia/reperfusion injury of skeletal muscle. *Lab. Invest.* 80:423-431.

13. Bissonnette, E.Y., Hogaboam, C.M., Wallace, J.L. and Befus, A.D. 1991. Potentiation of tumor necrosis factor-alpha-mediated cytotoxicity of mast cells by their production of nitric oxide. *J. Immunol.* 147:3060-3065.

14. Salvemini, D., Masini, E., Anggard, E., Mannaioni, P.F. and Vane, J. 1990. Synthesis of a nitric oxide-like factor from L-arginine by rat serosal mast cells: stimulation of guanylate cyclase and inhibition of platelet aggregation. *Biochem. Biophys. Res. Commun.* 169:596-601.

15. Gaboury, J.P., Niu, X.F. and Kubes, P. 1996. Nitric oxide inhibits numerous features of mast cell-induced inflammation. *Circulation* 93:318-326.

16. Deschoolmeester, M.L., Eastmond, N.C., Dearman, R.J., Kimber, I., Basketter, D.A. and Coleman, J.W. 1999. Reciprocal effects of interleukin-4 and interferon-gamma on immunoglobulin E-mediated mast cell degranulation: a role for nitric oxide but not peroxynitrite or cyclic guanosine monophosphate. *Immunology* 96:138-144.

17. Befus, A.D. and Bienenstock, J. 1979. Immunologically mediated intestinal mastocytosis in Nippostrongylus brasiliensis-infected rats. *Immunology* 38: 95-101.

18. Lee, T.D., Shanahan, F., Miller, H.R., Bienenstock, J. and Befus, A.D. 1985. Intestinal mucosal mast cells: isolation from rat lamina propria and purification using unit gravity velocity sedimentation. *Immunology* 55: 721-728.

19. Gilchrist, M., MacDonald, A.J., Neverova, I., Ritchie, B. and Befus, A.D. 1997. Optimization of the isolation and effective use of mRNA from rat mast cells. *J. Immunol. Methods* 201:207-214.

20. Nuovo, G.J. 1992. PCR In Situ Hybridization: Protocols and Applications. Raven Press, New York, NY. 247-282.

21. Nohara, O., Gilchrist, M., Dery, R.E., Stenton, G.R., Hirji, N.S. and Befus, A.D. 1999. Reverse transcriptase in situ polymerase chain reaction for gene expression in rat mast cells and macrophages. *J. Immunol. Methods* 226: 147-158.

22. Schmidt, H.H. and Kelm, M. 1996. Determination of nitrite and nitrate by the Griess reaction. In *Methods in Nitric Oxide Research*, (J.S. Stamler, ed.) J.W. Wiley and Sons, New York, NY. 491-498.

23. Cifone, M.G., D'Alo, S., Parroni, R., Millimaggi, D., Biordi, L., Martinotti, S. and Santoni, A. 1999. Interleukin-2-activated rat natural killer cells express inducible nitric oxide synthase that contributes to cytotoxic function and interferon-gamma production. *Blood* 93:3876-3884.

24. Hogaboam, C.M., Befus, A.D. and Wallace, J.L. 1993. Modulation of rat mast cell reactivity by IL-1 beta. Divergent effects on nitric oxide and platelet-activating factor release. *J. Immunol.* 151:3767-3774.

25. Ajuebor, M.N., Virag, L., Flower, R.J., Perretti, M. and Szabo, C. 1998. Role of inducible nitric oxide synthase in the regulation of neutrophil migration in zymosan-induced inflammation. *Immunology* 95:625-630.

26. Eastmond, N.C., Banks, E.M. and Coleman, J.W. 1997. Nitric oxide inhibits IgE-mediated degranulation of mast cells and is the principal intermediate in IFN-gamma-induced suppression of exocytosis. *J. Immunol.* 159:1444-1450.

27. Wills, F.L., Gilchrist, M. and Befus, A.D. 1999. Interferon-gamma regulates the interaction of RBL-2H3 cells with fibronectin through production of nitric oxide. *Immunology* 97: 481-489.

28. Abraham, S.N. and Arock, M. 1998. Mast cells and basophils in innate immunity. *Semin. Immunol.* 10:373-381.

29. Brooks, A.C., Whelan, C.J. and Purcell, W.M. 1999. Reactive oxygen species generation and histamine release by activated mast cells: modulation by nitric oxide synthase inhibition. *Br. J. Pharmacol.* 128:585-590.

30. Bissonnette, E.Y., Chin, B. and Befus, A.D. 1995. Interferons differentially regulate histamine and TNF-alpha in rat intestinal mucosal mast cells. *Immunology* 86:12-17.

31. Bissonnette, E.Y. and Befus, A.D. 1990. Inhibition of mast cell-mediated cytotoxicity by IFN-alpha/beta and -gamma. *J. Immunol.* 145:3385-339

Chapter 3

Tetrahydrobiopterin: Critical Factor in the Production and Role of Nitric Oxide in Mast Cells.

Authors, M. Gilchrist, C. Hesslinger and A.D. Befus. A version of this chapter was published in the *Journal of Biological Chemistry*. 2003. I contributed to this work by designing and carrying out all the experiments and writing the manuscript.

Introduction

Mast cells (MC) are immune effector cells located at strategic tissue sites, juxtaposed to vessels, epithelium and nerves. These phenotypic and functionally heterogeneous cells release granule-associated mediators (eg. histamine) and various newly-formed molecules (eg. phospholipid metabolites, cytokines) that play roles in innate and adaptive immune responses (1). Classically, MC can be activated via crosslinking of Fcε-bound IgE molecules by specific antigen (Ag), and inhibition of MC activation is a key component in controlling allergic diseases such as asthma (2). The T_H1 cytokine interferon-γ (IFN-γ) inhibits MC function, and dysregulation of IFN-γ contributes to atopy and asthma (2). IFN-γ modulation of cell phenotype is complex and involves regulation of gene expression (3) including the enzyme GTP-cyclohydrolase I (CHI), which produces tetrahydrobiopterin (BH_4) (4).

BH_4 is a critical factor in the production of the neurotransmitters serotonin, catecholamines, and other cellular activities including proliferation, cell-cycle regulation, and differentiation (4). The majority of cellular BH_4 is synthesized *de-novo* from GTP with CHI being rate-limiting (4). CHI activity is regulated at multiple levels, including transcription and phosphorylation, and can be inhibited by the CHI feedback regulatory protein (GFRP) (5,6). BH_4 is also an essential cofactor in nitric oxide (NO) formation from nitric oxide synthase (NOS), and increases in NOS transcription and activity need to be accompanied by a concurrent increase in BH_4 to sustain NO production (7).

NO is a reactive radical with pleotrophic effects with both physiological and pathological functions (8). NO is formed from L-arginine by the NOS family of isozymes that are subdivided on the basis of their Ca^{2+}-dependency and transcriptional inducibility. Ca^{2+}-dependent members include endothelial (eNOS) and neuronal (nNOS), characterized by constitutive expression and low NO production. Inducible NOS (iNOS) is upregulated by a variety of inflammatory mediators and functions independently of cellular Ca^{2+} levels and releases large amounts of NO (8).

MC functions including degranulation and adhesion, can be regulated by exogenous NO (9). We have recently shown that rat peritoneal MC (PMC) also constitutively express eNOS and can upregulate iNOS and produce NO (10). More recent *in-vivo* studies have

implicated MC as a significant source of NO in vascular tissues, likely through nNOS (11). MC produce low amounts of NO implying a paracrine, intracellular function. However, the roles for MC-derived NO remain poorly defined. Furthermore, our previous study showed diverse stimuli induced differing levels of NO release from MC, despite similar induction of NOS (10). Clearly, other regulatory events are responsible for differences in the time-course, quantities and outcomes of MC-derived NO (12). As no data is available on the role that BH_4 plays in NO production by MC, we hypothesized that post-transcriptional regulation of NOS activity by BH_4 is a critical determinant in NO production by MC.

The purpose of this study was two fold. First, to characterize both short-term (<5 min) Ca^{2+}-dependent constitutive NOS (cNOS) activity and sustained NO formation (>18 h) in MC using the fluorescent NO-sensitive probe diaminofluorescein (DAF). Secondly, to investigate the effects of CHI activity and BH_4 levels in both short-term (<5 min) and sustained (>18 h) NO production on MC reactivity. We demonstrate for the first time that a proportion of rat PMC dynamically activate intracellular Ca^{2+}-dependent NOS activity (<5 min) after Ag stimulation, which inhibits degranulation. Furthermore, IFN-γ treatment enhances long-term (>18 h) intracellular NO production, which also inhibits MC degranulation. MC constitutively express CHI mRNA and protein and upon stimulation with IFN-γ, PMC upregulate CHI mRNA and protein with a concordant increase in GTP-CHI activity. IFN-γ also decreases GFRP mRNA levels. The final outcome of these events is an increased level of NO production after IFN-γ treatment. Thus, modulation of MC BH_4 levels controls NO formation, and influences MC degranulation.

Materials and Methods

Animals

Adult male Sprague-Dawley (Crl:CD (SD) BR) rats were obtained from Charles River Canada Inc. (Quebec, Canada). For experiments requiring antigen stimulation, rats were sensitized with L_3 larvae of *Nippostrongylus brasiliensis* 5 to 6 weeks before MC isolation (10). Experimental procedures were approved by the University Animal Care Committee and were in accordance with the guidelines of the Canadian Council on Animal Care.

Chemicals

The NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), NO donor S-nitrosoglutathione (SNOG), and CHI inhibitor 2,4-Diamino-6-hydroxypyrimidine (DAHP) were obtained from Calbiochem (San Diego, CA). The sepiapterin reductase (SR) inhibitor n-acetyl serotonin (NAS) was obtained from Sigma (Oakville, ON), the pterin donor L-sepiapterin was obtained from Cayman Chemical (Ann Arbor, MI) and the NO probe 4,5-diaminofluorescein diacetate (DAF-FM) and Ca^{2+}-sensitive dye FURA-2, were obtained from Molecular Probes (Eugene, OR).

Antigen

Antigen (Ag) used to activate *in-vivo* sensitized PMC was a collection of soluble excretory and secretory products of the nematode *N. brasiliensis*, prepared as previously described (13). The antigen concentration was described as worm equivalents (WE) per mL.

MC isolation and stimulation

PMC were obtained by peritoneal lavage followed by centrifugation through gradient Percoll as previously described (10). PMC purity was >98% as determined by staining with toluidine blue. Cell viability was >99%. For most experiments PMC from unsensitized rats were treated with medium containing IFN-γ (200 U/mL)(Gibco/BRL). For studies of MC secretion, PMC from *N. brasiliensis* sensitized rats were treated with Ag (5 WE/mL).

Live-cell fluorescence determination of intracellular NO and Ca^{2+} production

NO production by PMC was assayed using DAF-FM, a cell-permeable NO-sensitive fluorescent dye (14). Coverslip-bottomed petri dishes (Falcon) were coated with rat plasma fibronectin (10 μg/mL)(Sigma) in PBS, pH 7.2, for 1 h at 37°C. PMC were loaded with 10 μM of DAF-FM, for Ca^{2+} experiments, PMC were loaded with FURA-2 (5μM), then incubated for 1 h at 37°C in the dark. Cells were resuspended in RPMI-1640 without phenol red and placed in dishes then incubated for 1 h at 37°C before use. Cell images were obtained using a Zeiss confocal laser scanning microscope (LSM510; Heidelberg, Germany) using a 488 nm (excitation) and 505-530 nm (emission) filter set for DAF and 340 nm (excitation) and 530 nm (emission) for FURA-2, with a 40x 1.3 oil Plan-Neofluar objective that was maintained at a constant 37°C with a heating ring. Ag

(5 WE/mL) was added to the cells and DAF/FURA-2 fluorescence intensity was determined in real-time, with 1 sec exposures obtained every 5 sec to avoid photobleaching. As DAF fluorescence is almost linear with NO concentration, quantitative analysis of each cell (8-15 cells per experiment) was obtained by averaging the peak relative fluorescent intensity (optical density arbitrary units) (15). The percent change in fluorescence from baseline (ΔF) was calculated by the equation $\Delta F=(F_b-F/F_b)$ x 100, where F_b is the basal fluorescence obtained from the frame before addition of stimulus and F is the fluorescence obtained after activation. Cells were defined as degranulating when they had ruffled membranes and had visibly extruded granules as previously described (16). Frame number and time reference were calculated using frame-by-frame analysis with MetaFluor imaging software (Universal Imaging Co, Downingtown, PA). In some experiments the NOS inhibitor L-NAME, or the NO donor SNOG was added 30 minutes before loading with DAF as negative and positive controls respectively.

Reverse transcription (RT)-PCR

Total RNA was isolated from PMC and pretreated with heparinase I (Sigma Chemical Co., St. Louis, MO) as previously described (17). RT-PCR was performed on a PTC-100 Thermal Cycler (MJ Research, Boston, MA). The primers were designed to be intron-spanning based on published sequence data: CHI, sense 5′-GATACCAGGAGACCATCTCA-3′, antisense 5′-TAGCATGGTGCTAG TGACAG-3′ (PCR product, 370 bp); GFRP, sense 5′-CCACTCACCATGCCCTACCT-3′, antisense 5′-GCAGCAAGGTTCCTGAGGCT-3′ (PCR product, 369 bp). The conditions for PCR amplification were as follows: denaturing at 95°C for 45 s, annealing for 45 s, and extension at 72°C for 1 min. The optimized annealing temperature was 48°C for CHI and 50°C for GFRP. Products were run on a 1.2 % agarose gel and stained with ethidium bromide (Sigma).

Cloning and sequencing of cDNA bands

The amplified PCR products were subcloned into pCR2.1™ plasmid vector using the T/A cloning kit (Invitrogen, Carlsbad, CA). Plasmid DNA was isolated with the GenElute™ Plasmid-isolation kit (Sigma). Double-stranded DNA sequencing was performed using M13 forward and reverse primers. Sequencing was conducted using

an ABI 373A automated sequencer (Applied Biosystems, Foster City, CA) by a dideoxy-chain termination method.

Semi-quantitative RT-PCR using an external standard

The linearized plasmid DNA containing the cloned inserts was used as a homologous amplification standard for semi-quantitative RT-PCR. Absorbance measurements at 260 nm were used to calculate the number of copies/μL. Known dilutions of the standard were amplified in concert with the unknown samples, and separated by agarose electrophoresis. Band densitometry (SigmaGel, SPSS Science, Chicago, IL) was used to compare the intensities of the standards to the unknowns to obtain the relative number of copies/μL.

Western blot analysis

PMC were incubated in 24 well plates at 1 x 10^6 cells/well from 0 to 18 h in various experimental conditions. The cells were dissociated in RIPA buffer (PBS,1% NP-40). The total protein content was determined by the Bradford technique (BioRad). Fifteen μg of protein from each sample was mixed with Lamelli buffer containing SDS and β-mercaptoethanol. Samples were separated on a 12% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (BioRad). CHI was identified with rat monoclonal anti-CHI (6H11) (I.Ziegler, Munchen, Germany) (5) diluted (1 μg/mL) and HRP conjugated goat anti-rat IgG (0.05 μg/mL)(Serotec, Raleigh, NC). Labelling was detected by chemiluminescence with SuperSignal substrate solution (Pierce, Rockford, IL). The resulting bands were scanned and quantified in a gel scanner (ImageMaster DTS, Pharmacia).

Immunofluorescence detection of CHI

Localization of CHI in PMC was performed on PMC fixed in 4% paraformaldehyde for 20 min, then permeablized with 0.1% triton X-100 in PBS. Slides were blocked for 30 min with 10% FBS and 3% BSA in PBS, then incubated overnight at 4°C with 1 μg/mL of rat monoclonal CHI antibody (6H11). Specific antibody binding was detected with rhodamine red-labeled goat anti-rat antibody (Molecular Probes, Eugene, OR). Negative controls labeled with isotype (rat IgG1) antibody were run concurrently.

Enzyme assay for CHI activity

The activity of CHI was determined on homogenized cell extracts as previously described (5). Briefly, the reaction product was oxidized to neopterin triphosphate by acidic iodine solution. After reduction of excess iodine by ascorbic acid, the sample was immediately separated by ion pair reverse-phase HPLC. Neopterin was then determined by reverse-phase HPLC.

Measurement of NO_2^- production

NO_2^- in culture (phenol red free) supernatants was measured by the Griess reaction (10).

β-hexosaminidase (β-hex) assay

β-hex release was investigated as a functional measure of the BH_4/NO effects on granule mediator secretion as has been previously described (18).

Statistical analysis

All experiments were performed at least three times. Data was analyzed using analysis of varience (ANOVA) followed by the Bonferroni test for comparisons. P values < 0.01 were considered significant.

Online Supplemental Material

Real-time images of degranulation and NO production in Ag/IgE-stimulated MC are shown in the online supplemental video 1. The video image was obtained over a 4 min (240 sec) period, with 1 sec exposures every 5 sec. The video was compressed 10X using Adobe Premiere (Adobe Systems Canada, Ottawa, ON). Images were captured using a 40X objective on a Leica confocal laser scanning microscope. Video 1 shows a flux of green DAF fluorescence in a proportion of MC, which is correlated with inhibition of degranulation. MC that are negative for DAF degranulate quickly.

Results

Sustained (>18 h) intracellular production of NO in IFN-γ-treated PMC

To confirm that IFN-γ treated PMC produced NO we utilized the NO specific fluorescent molecule DAF. Confocal microscopic analysis showed that IFN-γ caused an increase in intracellular DAF fluorescence after 18 h of treatment compared to controls. The DAF fluorescence localized to cytoplasmic and occasionally to perinuclear sites (Fig. 3.1A).

Real-time confocal analysis of IgE-mediated degranulation and NO production

Next we wanted to assess constitutive NOS activity as there is evidence that MC possess cNOS activity (19). To study this short-term (<5 min) NOS activity, its modulation and association with degranulation we developed a real-time assay to detect DAF fluorescence and analyze dynamic changes in NO production by individual cells. PMC were isolated and loaded with DAF (10 μM). A large proportion of unstimulated PMC showed weak DAF fluorescence, with an occasional cell (< 2%) having stronger cytoplasmic fluorescence. PMC showed no visible signs of degranulation, as their membranes were intact and showed no signs of granule release as noted in other MC live cell studies (16).

Stimulation with Ag (5 WE/mL) caused an increase in the number of degranulating cells. Degranulating PMC did not show any increases in DAF fluorescence (Fig. 3.1B and on Video 1). Interestingly a proportion (33.1±2.2%, mean±SEM of 116 total cells in eight different experiments) of PMC showed an immediate (<2 min) increase in NO production (DAF fluorescence) and no visible signs of degranulation. DAF fluorescence accumulated in the cytoplasm of these cells, and a population of strong NO producing cells showed intense nuclear positivity (Fig. 3.1B and on Video 1). While the strong DAF fluorescence cells showed no sign of degranulation after 60 min of observation, some of the less positive cells eventually did show degranulation. Regression analysis comparing these weaker DAF fluorescent PMC to time of degranulation showed a highly significant correlation (R^2=0.7056, $p < 0.001$) (Fig. 3.1C). Equal loading of PMC with DAF was confirmed by adding the NO donor SNOG (100 μM); after 20 min almost all PMC showed strong cytoplasmic staining with DAF (data not shown). Interestingly, no nuclear localization of DAF fluorescence was seen after adding this exogenous NO source. Specificity of the effect was determined by pretreating PMC with the NOS inhibitor L-NAME (100 μM); these cells showed no flux of DAF fluorescence (data not shown). These results support the hypothesis that PMC possess cNOS activity that can be stimulated in the short-term (<5 min) in a proportion of cells by IgE crosslinking. Our data also suggest that immediate NO production by PMC causes inhibition of mediator release.

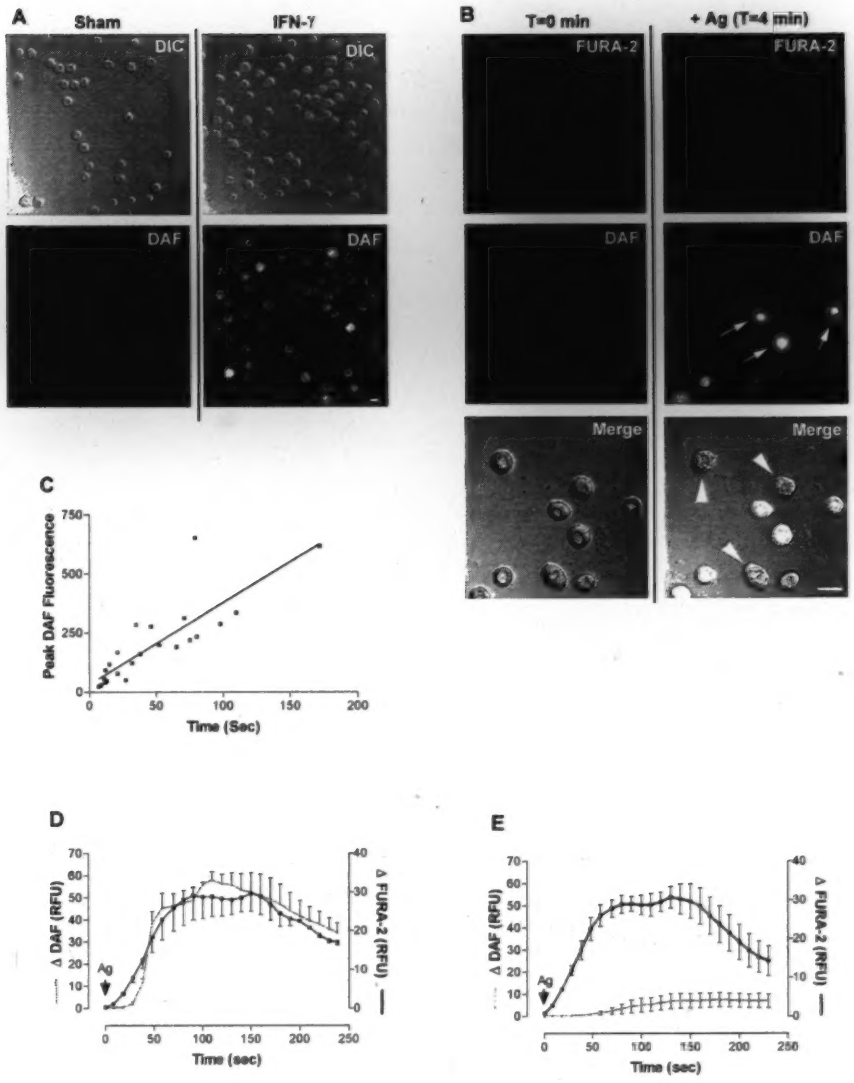

A
Sham
IFN-γ
DIC
DIC
DAF
DAF
B
T=0 min
+ Ag (T=4 min)
FURA-2
FURA-2
DAF
DAF
Merge
Merge
C
Peak DAF Fluorescence
750
500
250
0
0
50
100
150
200
Time (Sec)
D
Δ DAF (RFU)
Δ FURA-2 (RFU)
Ag
70
60
50
40
30
20
10
0
40
30
20
10
0
0
50
100
150
200
250
Time (sec)
E
Δ DAF (RFU)
Δ FURA-2 (RFU)
Ag
70
60
50
40
30
20
10
0
40
30
20
10
0
0
50
100
150
200
250
Time (sec)

Fig. 3.1. *A,* Fluorescence detection of intracellular nitric oxide (NO) production in interferon-γ (IFN-γ)-treated rat peritoneal mast cells (PMC). Cells were incubated with IFN-γ (200 U/mL) for 18 h, then stained with diaminofluorescein (DAF) for 20 min. DAF fluorescence (green) was visualized by confocal analysis. Untreated PMC are used as the control. Differential interference contrast (DIC) shows cellular morphology. Original magnification X 400, bar=10 μm. Representative of four independent experiments. *B*, Real-time confocal analysis of Ca^{2+} accumulation and NO production in antigen (Ag)-stimulated PMC. PMC from sensitized rats were loaded with both DAF (10 μM) and FURA-2 (5 μM) for 1 h. Cells were stimulated with Ag and images simultaneously obtained every 5 s from both DAF and FURA-2 channels. The panels show representative DAF (green), FURA-2 (red) and combined images (yellow) obtained before the addition of Ag (t=0) and at a time point when no further changes in PMC morphology were observed (t=4 min). White arrowheads indicates degranulating cells, white arrow indicated nuclear accumulation of DAF in PMC nucleus. Original magnification X 600, bar=10 μm. Results are representative of three independent experiments. Complete movie (Video 1) is available as supplementary information. *C,* Correlation between NO production (peak DAF fluorescence; arbitrary units) and time to degranulation (sec) from Ag stimulated MC that produce NO but later show signs of degranulation. The data shown are from 30 individual MC from five independent experiments. Linear regression analysis was used, and the correlation is highly significant ($p < 0.001$). *D,* Temporal relationship between Ca^{2+} and NO in PMC after Ag stimulation. Changes in the average pixel intensity (Δ) for both DAF (NO; gray line) and FURA-2 (Ca^{2+}; black line) in non-degranulating PMC were plotted vs time (sec) after addition of Ag. Each line represents the average change in fluorescence (mean ± SEM) for 3-7 cells from one field of a representative experiment. *E,* Changes in the average pixel intensity (Δ) for both DAF (NO; gray line) and FURA-2 (Ca^{2+}; black line) in degranulating PMC were plotted vs time (sec) after addition of Ag. Each line represents the average change in fluorescence (mean ± SEM) for 3-7 cells from one field of a representative experiment. Results are representative of three separate experiments. Data expressed as relative fluorescence units (RFU).

Ca^{2+} flux and NO production in PMC

The results above may involve constitutive NOS (eNOS or nNOS) activity in PMC (10). As both eNOS and nNOS are Ca^{2+}-dependent we wished to determine if increases in intracellular Ca^{2+} precede the flux of NO seen in Ag/IgE-treated PMC. PMC were isolated and loaded with both DAF (10 μM) and the Ca^{2+}-sensitive dye FURA-2 (5 μM). As has been previously shown, upon stimulation with Ag (5 WE/mL) all PMC showed an increase in FURA-2 fluorescence beginning within 30 sec (Fig. 3.1B)(20). DAF fluorescence in strong NO producing cells was delayed by almost 20 sec on average compared to FURA-2 (Fig. 3.1D). However, DAF fluorescence in degranulating MC showed little change (Fig. 3.1E) Interestingly, while PMC were heterogeneous in their DAF positivity, all cells were homogenous in their Ca^{2+} flux (Fig. 3.1D&E). This indicates that Ca^{2+} flux precedes short-term NO production in non-degranulating, Ag-treated PMC.

GTP-CHI/GFRP mRNA expression in PMC

We recently showed that rat PMC treated with IFN-γ could be stimulated to express iNOS mRNA (10), however, no data exists concerning the expression of CHI or GFRP in *in-vivo*-derived MC. mRNA production was thus determined using RT-PCR with gene-specific primers. PMC constitutively express significant CHI and GFRP mRNA. (Fig.3.2A&B). All PCR reactions were negative when the RT step was eliminated, indicating that there was no contamination from genomic DNA (data not shown). Cloned PCR amplicons were sequenced and showed >98% identity with published sequences by BLAST (NCBI) analysis.

Differential regulation of GTP-CHI and GFRP mRNA

Since stimuli that increase NOS expression often induce a concordant increase in de novo BH_4 production (7), we looked at CHI and GFRP mRNA regulation by IFN-γ. Total RNA was extracted from unstimulated PMC (>98% pure), and from PMC treated *in vitro* with IFN-γ (200 U/mL). CHI and GFRP mRNA production was assessed by semiquantitative RT-PCR vs a standard curve constructed with known copy numbers of cloned inserts amplified under identical PCR conditions. Within 2 h following treatment with IFN-γ the CHI signal in PMC increased approximately 3-fold. Levels of CHI mRNA reached maximal expression at 6 h and continued to increase to 18 h (Fig.

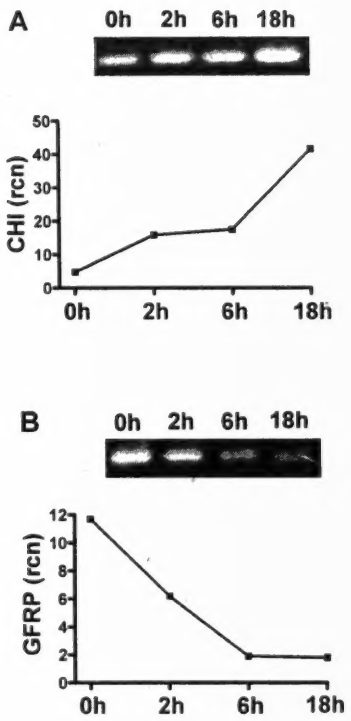


Fig. 3.2. Semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of GTP-cyclohydrolase I (CHI) (A) and GTP-cyclohydrolase feedback regulatory protein (GFRP) (B) mRNA expression in rat peritoneal mast cells (PMC) treated with interferon-γ (IFN-γ)(200 U/mL). RNA from IFN-γ-treated PMC were isolated at indicated time points and analyzed by RT-PCR vs known concentrations of homologous cloned standard. Band densitometries from the standards were used to determine the relative copy number (rcn) in each of the IFN-γ-treated samples. Graphic representation of the time-course with responses plotted as rcn. Data are from one representative experiment. Similar results were seen with three separate batches of PMC (n=3).

3.2A). No change in CHI mRNA was noted in unstimulated PMC at similar time points (data not shown). GFRP mRNA expression from IFN-γ-treated PMC was significantly down-regulated beginning at 2 h and continued until at least 18 h of treatment (Fig.3.2B). Results are from PMC RNA obtained from three independent batches of cells.

CHI protein expression

To further evaluate if the actions of IFN-γ on GTP-CHI mRNA levels were extended to protein expression, Western blot analysis was employed. Unstimulated PMC constitutively produced a band of 30 kDa detected by the antibody 6H11 (Fig. 3.3A). Exposure of PMC to IFN-γ produced a significant increase in CHI protein expression, with maximal stimulation between 6-18 h. Again unstimulated PMC cultured during the same time showed no increase in CHI protein expression. Furthermore, PMC stimulated with Ag/IgE also showed no detectable change in protein expression (data not shown).

Immunofluorescence localization of CHI in rat MC

As the intracellular localization of CHI is poorly understood we used confocal laser scanning microscopy to assess the cellular expression pattern of CHI protein in PMC. Unstimulated PMC from naïve rats showed a clear plasma membrane pattern with some nuclear immunofluorescence in occasional cells. Membrane staining was confirmed by imaging en face 0.5 μm sections through each cell. This stacked series of multiple images was deconvoluted and 3-D reconstructed using Zeiss LSM 510 software (Heidelberg, Germany). PMC stimulated with IFN-γ on the other hand showed a pronounced increase in cytosolic immunofluorescence while maintaining membrane and some nuclear labeling (Fig.3.3B).

CHI enzymatic activity

To determine the functional consequences of IFN-γ treatment we determined CHI enzymatic activity, employing an HPLC based assay as previously described (5). Untreated PMC were compared with cells treated with IFN-γ or Ag/IgE. In unstimulated cells, CHI activity was low (< 5.0 nmol/mg/min) as previously described (5) but was markedly elevated upon treatment with IFN-γ with a significant increase

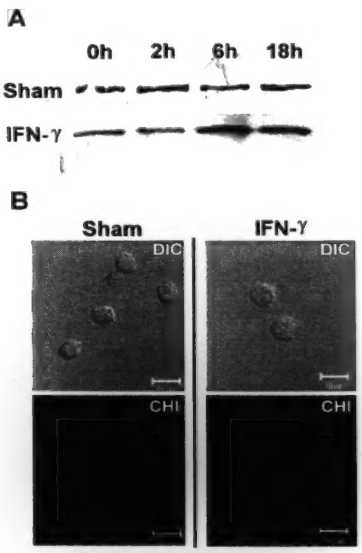


Fig. 3.3. *A,* Western blot analysis using anti-GTP-cyclohydrolase I (CHI) antibody (6H11) with 15 μg of total cell lysate obtained from rat peritoneal mast cells (PMC) treated with interferon-γ (IFN-γ)(200 U/mL) for the indicated times. As a control, lysates from untreated PMC incubated for identical times were also analysed. Results are representative of three separate experiments. *B,* Confocal analysis of CHI localization in sham control and IFN-γ-treated PMC. Differential interference contrast (DIC) shows cells in the field, CHI immunofluorescence shown in red. CHI is detected in the plasma membrane via imaging 0.5 μm optical en face sections throughout the cell. Original magnification X 800, bar = 10 μm.

observed at 6 h that continued to at least 18 h (Fig. 3.4). As expected from western blot data, no increase in CHI activity was seen after Ag/IgE treatments (data not shown).

Control of PMC NO (>18 h) production by BH_4

Previously, PMC have been shown to produce NO (10). Unstimulated PMC spontaneously released low levels of NO_2^- (0.27±0.03 μM). Activation with IFN-γ caused PMC to produce significantly greater ($p<0.01$) amounts of NO_2^- (3.6±0.2 μM). (Fig.3.5). To further define the role of CHI and BH_4 in MC NO production we employed the pharmacological inhibitor of CHI (DAHP)(4). Addition of DAHP along with IFN-γ for 24 h inhibited NO production in a dose-dependent manner (Fig.3.5). To further confirm that PMC cellular BH_4 levels were limiting in NO production, cells were treatment with an exogenous substrate for BH_4 production, sepiapterin. Sepiapterin treatment slightly (but not significantly) potentiated NO production in untreated cells, and increased nitrite accumulation upon IFN-γ stimulation (4.6±0.3 μM) (Fig.3.5). Significant inhibition ($p<0.01$) was attained with DAHP concentrations as low as 100 μM, with nitrite levels at or below sham values with concentrations of 500 μM. As DAHP at high concentrations (500 μM) completely inhibits nitrite formation, there appears to be little contribution via the salvage pathway under these conditions. These results were confirmed using the SR inhibitor NAS (data not shown). However, co-administration of DAHP (500 μM) with 200 μM sepiapterin, significantly ($p<0.01$) reconstituted nitrite production (3.2±0.1 μM) (Fig. 3.5).

Effects of BH_4 modulation (>18 h) on MC degranulation

Given the above results showing that BH_4 levels are important in regulating MC NO production, we investigated the effects of IFN-γ and modulation of BH_4 on MC degranulation by measuring β-hex release. PMC were treated for 18 h with IFN-γ (200 U/mL), DAHP (500 μM) or sepiapterin (200 μM) then stimulated with Ag (5 WE/mL) for 30 min. As Fig. 3.6A shows, Ag stimulation induced 21±1.2 % release, while IFN-γ treatment significantly ($p<0.05$) inhibited Ag induced release by over 40% (11±1.5 % release). Pretreatment of PMC with DAHP (500 mM) for 24 h resulted in a significant ($p<0.05$) increase in Ag-induced β-hex release (from 21±1.2% to 37±2.3%) and abrogated the inhibitory effect of IFN-γ. Treatment with sepiapterin induced

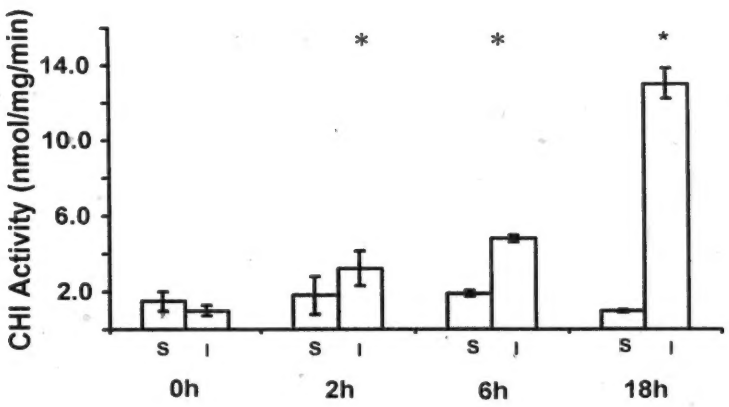


Fig. 3.4. Time course analysis of GTP-cyclohydrolase I (CHI) enzymatic activity in rat peritoneal mast cells (PMC) cultured with (I) or without (S) IFN-γ (200 U/mL). PMC were incubated for the indicated times and cell pellets obtained. Data is shown as nmol/mg/min (mean±SEM of three separate experiments). *, Indicates $p < 0.01$ by comparison with untreated cells.

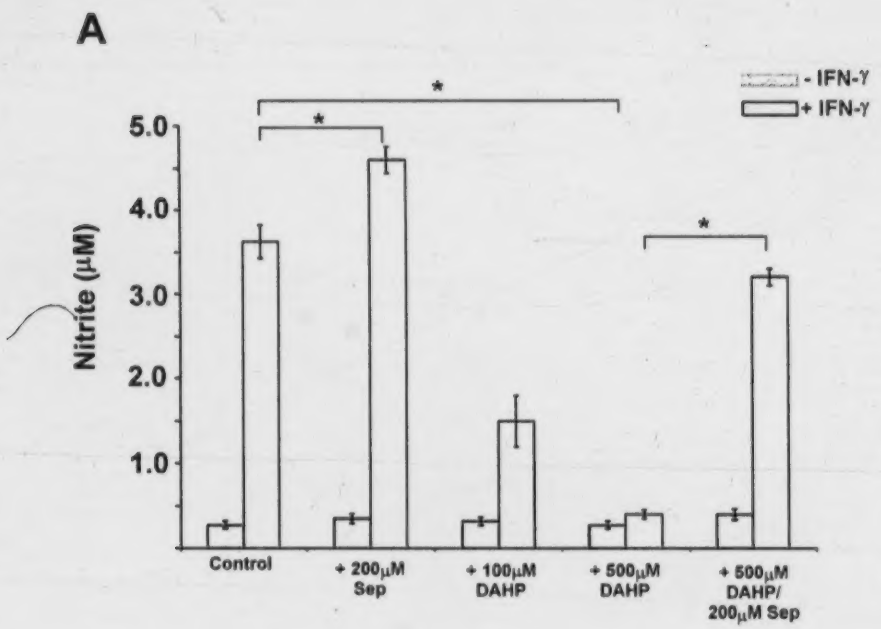


Fig.3.5. The effect of tetrahydrobiopterin (BH_4) modulation on rat peritoneal mast cells (PMC) nitric oxide (NO) production (nitrite) measured by the Griess assay. PMC treated with the alternate pterin substrate, sepiapterin (Sep)(200 μM) were used to determine if increased BH_4 availability effects NO production. Cells treated with interferon-γ (IFN-γ) and the GTP cyclohydrolase I (CHI) inhibitor 2,4-diamino-6-hydroxypyrimidine (DAHP)(100 μM and 500 μM) were used to determine the effect of decreased BH_4 levels on NO production. Addition of sepiapterin (200 μM) was used to reconstitute BH_4 levels in DAHP-treateed PMC. Cells from unsensitized rats were used as the sham control. Data shown as mean ± SEM of three experiments, 2 x 10^5 cells/sample were analyzed. *, Indicates $p < 0.01$ by comparison with untreated cells.

significant ($p<0.05$) inhibition of β-hex release after Ag stimulation in IFN-γ and untreated PMC. Furthermore, modulation of BH_4 levels also influenced spontaneous β-hex release (Fig. 3.6B). PMC spontaneously release about 6% (6.3±0.9%) β-hex. DAHP pretreatment significantly ($p<0.01$) increased spontaneous β-hex release (16.1±1.3%). Interestingly, sepaipterin also caused a modest but not significant inhibition of spontaneous release (Fig. 3.6B).

Effects of BH_4 inhibition on short-term (<5 min) NO production/MC degranulation

The results obtained above indicated that DAHP and sepiapterin modulate MC NO production long term (>18 h) and thus regulate degranulation. As BH_4 is a critical co-factor for all NOS isoforms, we studied the effects of DAHP on Ca^{2+}-dependent NOS activity and degranulation. PMC from sensitized rats were isolated and loaded with DAF (10 μM), some cells were first pre-treated with DAHP (500 μM) for 6 h before loading. PMC were then treated with Ag and analyzed by real-time confocal microscopy as outlined above. DAHP treated MC showed increased membrane changes, with some degranulating MC clearly evident (Fig. 3.7). Furthermore, DAHP treated PMC showed no NO flux compared to untreated controls and most cells degranulated in < 3 min.

Discussion

NO is a known modulator of both pro- and anti-inflammatory MC functions though regulation of its endogenous production is unknown (9). These diverse effects imply a complex regulatory mechanism to control the timing, amounts and location of NO production (8). As BH_4 is a critical NOS cofactor, we investigated the expression and regulation of BH_4 production in MC and its role in regulating long- (inducible) and short-term (constitutive) NOS activity and resulting effect on MC secretion.

Using the fluorescent NO marker, DAF, we identified both long (iNOS) and short-term (cNOS) intracellular NO production in PMC. We have previously shown iNOS upregulation in PMC following IFN-γ treatment (10). DAF staining in IFN-γ (long-term) treated PMC showed a diffuse cytoplasmic pattern with possible Golgi staining in some cells. Earlier studies by ourselves and others have indicated that PMC may also have Ca^{2+}-dependent NOS activity by eNOS or bNOS (10,11). A proportion of PMC treated with Ag/IgE showed immediate (short-term) NO formation preceded by a flux in

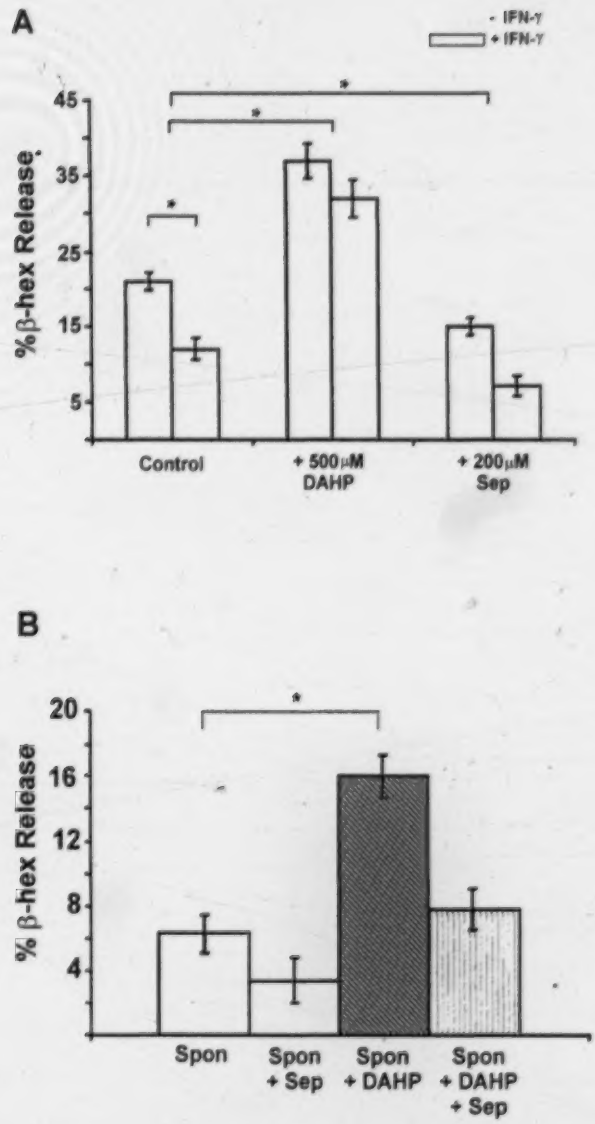


Fig. 3.6. *A,* Effects of tetrahydrobiopterin (BH_4) availability on β-hexosaminidase (β-hex) release from rat peritoneal mast cells (PMC). PMC from sensitized rats were cultured for 18 h with (open bars) and without (shaded bars) interferon-γ (IFN-γ)(200 U/mL) then stimulated with antigen (Ag) for 20 min and β-hex release measured. PMC were also treated ± IFN-γ in the presence of 2,4-diamino-6-hydroxypyrimidine (DAHP)(500 μM) or sepiapterin (Sep)(200 μM) for 18 h and then stimulated with Ag for 20 min. *B,* Effects of BH_4 modulation on spontaneous β-hex release. PMC were treated for 18 h in the presence of DAHP (500 μM), sepiapterin (200 μM) or both and spontaneous release of β-hex was measured and compared to release from untreated PMC. Data shown as mean ± SEM of three experiments. *, Indicates $p < 0.01$.

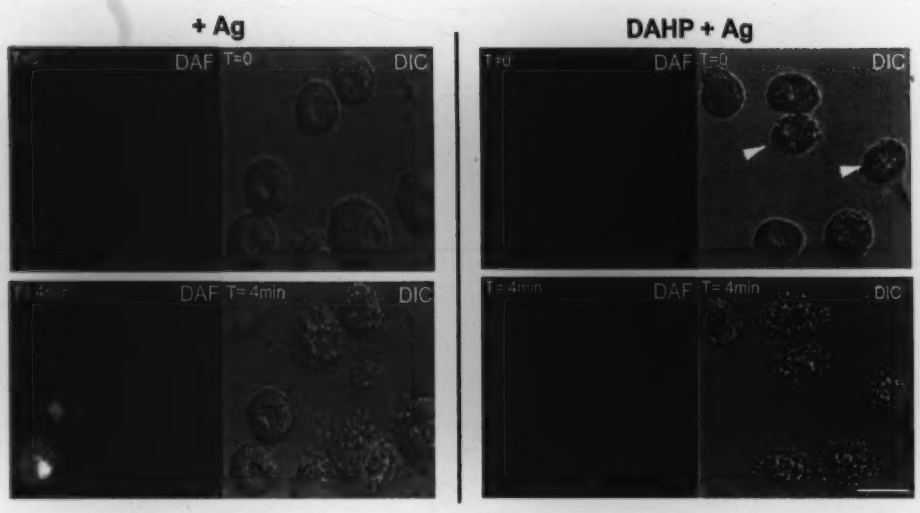


Fig. 3.7. Real-time confocal analysis of the effects of tetrahydrobiopterin (BH_4) inhibition on antigen (Ag) induced nitric oxide (NO) production and degranulation. Peritoneal mast cells (PMC) from sensitized rats were treated ± 2,4-diamino-6-hydroxypyrimidine (DAHP)(500 μM), loaded with diaminofluorescein (DAF)(10 μM), and stimulated with Ag. Live images of Ag-stimulated PMC were obtained every 5 sec. The panels show representative DAF fluorescence (green) and combined (DAF + differential interference contrast (DIC)) images obtained before the addition of Ag (t=0) and at a time point where no further changes in PMC morphology were observed (t=4 min). White arrowheads indicate degranulating cells. Original magnification X 600, bar=10 μm. Results are representative of eight separate experiments.

Ca^{2+} levels. As both cNOS isoforms are Ca^{2+}-dependent for their activity, our results support a functional cNOS in MC.

IFN-γ is an important T_H1 cytokine and is a potent inhibitor of T_H2 type responses including those involving MC. Our data shows a significant (8-fold) potentiation of CHI mRNA expression upon treatment with IFN-γ. As CHI is a well-known IFN-γ inducible gene, this data is consistent with that shown for other cells types (21,22). We also investigated the mRNA expression of a CHI regulatory protein, GFRP (23). IFN-γ significantly downregulates GFRP mRNA production in PMC which coincides with increased CHI production. By contrast, in C6 (glioblastoma) cells, IFN-γ or IL-1β caused an increase in CHI expression with no effect on GFRP, while in THP-1 (myelomonocytoma) cells, GFRP was unaltered by IFN-γ or IL-1β, but instead was downregulated by LPS (24,25). IFN-γ thus has a unique regulatory effect on PMC that potentiates BH_4 production through increased CHI and inhibiting GFRP expression.

The western blot data confirm that IFN-γ upregulates CHI protein expression in a similar manner. We also showed that IFN-γ treatment increased CHI enzymatic activity and BH_4 accumulation in PMC as seen in other cell types (21). This increased expression is associated with altered localization of CHI in PMC. In untreated PMC there is a distinct membranous localization of CHI with occasional nuclear positivity, but little cytoplasmic staining. Treatment with IFN-γ results in a pronounced increase in the cytoplasmic accumulation of CHI. Previous studies in neurons have shown both cytoplasmic and nuclear CHI staining (26). Our data are novel in that we show for the first time a membranous localization of CHI. A possible explanation for distinct localization patterns of CHI may be that BH_4 is highly labile *in-vivo*, and CHI may need to be in close proximity to NOS to maintain adequate BH_4 levels and NO production (4). As such, our immunohistochemistry results (10) and that from others have shown a diffuse cytoplasmic pattern of iNOS expression in MC (27), a similar intracellular location to our DAF results with IFN-γ. Thus colocalization of iNOS and CHI may contribute to increased NO production after IFN-γ treatment.

BH_4 is an important cofactor for all NOS isoforms. Using the CHI inhibitor DAHP and SR inhibitor NAS we demonstrated that diminishing BH_4 leads to decreased NO.

Decreased NO production through direct inhibition of NOS has been previously shown to potentiate MC degranulation (28). We found similar results, as inhibition of BH_4 production increased mediator release from PMC. Furthermore, the inhibitory effect of IFN-γ (18 h) treatment on PMC degranulation was removed by DAHP, implying that the IFN-γ effect was BH_4 dependent. Our results using DAHP and NAS are similar to those seen with mouse macrophages, though higher concentrations of inhibitor were necessary to inhibit NO in those studies (29-31). As both cell types constitutively express CHI this disparity may point to differences in compartmentalization of CHI and NOS (30).

We previously showed that NO production in naïve PMC likely relies on cNOS activity (10). PMC treated with DAHP, sepiapterin, or both, but without a known secretagogue showed modulation of degranulation. Therefore regulation of BH_4 levels is also important in cNOS activity in PMC. Our results using live cell analysis further support these findings as DAHP inhibited NO production and increased degranulation.

Levels of BH_4 are a determining factor in the reactive radicals derived from NOS as the NOS dimer may produce superoxide (O_2^-) or peroxynitrite ($ONOO^-$), rather than NO (32). Interestingly, O_2^- potentiates MC secretion, although the source of O_2^- is unknown (28). It is interesting to speculate that O_2^- arises in MC from NOS that is not saturated with BH_4. Indeed, previous studies using the NOS inhibitor L-NAME showed decreased NO and a resulting increase in O_2^- production and MC degranulation (28,33). Interestingly, L-NAME also inhibits the reductase region of the NOS enzyme, causing the preferential formation of O_2^- (34). Thus NOS may maintain a redox balance in MC by producing NO or ONOO to inhibit degranulation, and removal of BH_4 leads to O_2^- production and degranulation. This hypothesis requires further study.

Ag/IgE activation of MC causes a transient hyperphosphorylation of CHI and increase in cellular BH_4 (5) and may be an activation step leading to degranulation. Our live cell results add a caveate to this interpretation. As this BH_4 production coincides with NO production in MC after Ag/IgE. BH_4 levels may play a key role in controlling MC degranulation, and cellular localization of BH_4 and its role in NO production require further investigation.

The nuclear localization of NO is to our knowledge the first identification of dynamic accumulation of NOS activity in the nucleus. Previous studies show eNOS protein in nuclear and peri-nuclear regions (35,36). Furthermore, studies have shown both Ca^{2+} and calmodulin accumulation at nuclear sites in activated MC (20,37). As both Ca^{2+} and calmodulin are critical in NOS activity, there may be a nuclear/NOS axis, though a role for NO in the nucleus is unknown (38).

Live cell imaging has allowed the investigation of MC functional heterogeneity and NO production at the single cell level. Furthermore, the differing localization of DAF positivity in IFN-γ and Ag/IgE treated MC implies involvement of distinct regulatory mechanisms. This pattern of DAF positivity was divergent from that seen when DAF loaded MC were treated with an NO donor. Thus, interpretation of data in NO studies of MC function will have to carefully consider the source of NO as they may have potentially divergent outcomes and molecular mechanisms

In conclusion, we have characterized both short and long-term intracellular NO production in MC and showed that increased intracellular NO production is associated with inhibition of Ag-induced degranulation. Furthermore, BH_4 levels are critical to sustaining NO production in MC and can regulate MC degranulation. Further study of NO regulatory pathways such as through BH_4, will help define novel molecular targets that modulate MC-related inflammatory and innate responses.

References

1. Wedemeyer, J., Tsai, M., and Galli, S.J. 2000. Roles of mast cells and basophils in innate and acquired immunity. *Curr. Opin. Immunol.* 12:624-631.

2. Busse, W.W. and Rosenwasser, L.J. 2003. Mechanisms of asthma. *J. Allergy. Clin. Immunol.* 111:S799-S804.

3. Ramana, C.V., Gil, M.P., Schreiber, R.D., and Stark, G.R. 2002. Stat1-dependent and -independent pathways in IFN-gamma-dependent signaling. *Trends Immunol.* 23:96-101.

4. Thony, B., Auerbach, G., and Blau, N. 2000. Tetrahydrobiopterin biosynthesis, regeneration and functions. *Biochem. J.* 347 Pt 1:1-16.

5. Hesslinger, C., Kremmer, E., Hultner, L., Ueffing, M., and Ziegler, I. 1998. Phosphorylation of GTP cyclohydrolase I and modulation of its activity in rodent mast cells. GTP cyclohydrolase I hyperphosphorylation is coupled to high affinity IgE receptor signaling and involves protein kinase C. *J. Biol. Chem.* 273:21616-21622.

6. Milstien, S., Jaffe, H., Kowlessur, D., and Bonner, T.I. 1996. Purification and cloning of the GTP cyclohydrolase I feedback regulatory protein, GFRP. *J. Biol. Chem.* 271:19743-19751.

7. Togari, A., Arai, M., Mogi, M., Kondo, A., and Nagatsu, T. 1998. Coexpression of GTP cyclohydrolase I and inducible nitric oxide synthase mRNAs in mouse osteoblastic cells activated by proinflammatory cytokines. *FEBS. Lett.* 428:212-216.

8. Alderton, W.K., Cooper, C.E., and Knowles, R.G. 2001. Nitric oxide synthases: structure, function and inhibition. *Biochem. J.* 357:593-615.

9. Coleman, J.W. 2002. Nitric oxide: a regulator of mast cell activation and mast cell- mediated inflammation. *Clin. Exp. Immunol.* 129:4-10.

10. Gilchrist, M., Savoie, M., Nohara, O., Wills, F.L., Wallace, J.L., and Befus, A.D. 2002. Nitric oxide synthase and nitric oxide production in in vivo-derived mast cells. *J. Leukoc. Biol.* 71:618-624.

11. Kashiwagi, S., Kajimura, M., Yoshimura, Y., and Suematsu, M. 2002. Nonendothelial source of nitric oxide in arterioles but not in venules: alternative source revealed in vivo by diaminofluorescein microfluorography. *Circ. Res.* 91:e55-e64.

12. Grisham, M.B., Jourd'Heuil, D., and Wink, D.A. 1999. Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites:implications in inflammation. *Am. J. Physiol.* 276:G315-G321.

13. Lee, T.D., Shanahan, F., Miller, H.R., Bienenstock, J., and Befus, A.D. 1985. Intestinal mucosal mast cells: isolation from rat lamina propria and purification using unit gravity velocity sedimentation. *Immunology* 55:721-728.

14. Suzuki, N., Kojima, H., Urano, Y., Kikuchi, K., Hirata, Y., and Nagano, T. 2002. Orthogonality of calcium concentration and ability of 4,5- diaminofluorescein to detect NO. *J. Biol. Chem.* 277:47-49.

15. Itoh, Y., Ma, F.H., Hoshi, H., Oka, M., Noda, K., Ukai, Y., Kojima, H., Nagano, T., and Toda, N. 2000. Determination and bioimaging method for nitric oxide in biological specimens by diaminofluorescein fluorometry. *Anal. Biochem.* 287:203-209.

16. Williams, R.M., Shear, J.B., Zipfel, W.R., Maiti, S., and Webb, W.W. 1999. Mucosal mast cell secretion processes imaged using three-photon microscopy of 5-hydroxytryptamine autofluorescence. *Biophys. J.* 76:1835-1846.

17. Gilchrist, M., MacDonald, A.J., Neverova, I., Ritchie, B., and Befus, A.D. 1997. Optimization of the isolation and effective use of mRNA from rat mast cells. *J. Immunol. Methods* 201:207-214.

18. Kulka, M., Gilchrist, M., Duszyk, M., and Befus, A.D. 2002. Expression and functional characterization of CFTR in mast cells. *J. Leukoc. Biol.* 71:54-64.

19. Hogaboam, C.M., Befus, A.D., and Wallace, J.L. 1993. Modulation of rat mast cell reactivity by IL-1 beta. Divergent effects on nitric oxide and platelet-activating factor release. *J. Immunol.* 151:3767-3774.

20. Mori, S., Saino, T., and Satoh, Y. 2000. Effect of low temperatures on compound 48/80-induced intracellular Ca2+ changes and exocytosis of rat peritoneal mast cells. *Arch. Histol. Cytol.* 63:261-270.

21. Schott, K., Gutlich, M., and Ziegler, I. 1993. Induction of GTP-cyclohydrolase I mRNA expression by lectin activation and interferon-gamma treatment in human cells associated with the immune response. *J. Cell. Physiol.* 156:12-16.

22. Delgado-Esteban, M., Almeida, A., and Medina, J.M. 2002. Tetrahydrobiopterin deficiency increases neuronal vulnerability to hypoxia. *J. Neurochem.* 82:1148-1159.

23. Kapatos, G., Hirayama, K., Shimoji, M., and Milstien, S. 1999. GTP cyclohydrolase I feedback regulatory protein is expressed in serotonin neurons and regulates tetrahydrobiopterin biosynthesis. *J. Neurochem.* 72:669-675.

24. Werner, E.R., Bahrami, S., Heller, R., and Werner-Felmayer, G. 2002. Bacterial lipopolysaccharide down-regulates expression of GTP cyclohydrolase I feedback regulatory protein. *J. Biol. Chem.* 277:10129-10133.

25. Vann, L.R., Twitty, S., Spiegel, S., and Milstien, S. 2000. Divergence in regulation of nitric-oxide synthase and its cofactor tetrahydrobiopterin by tumor necrosis factor-alpha. Ceramide potentiates nitric oxide synthesis without affecting GTP cyclohydrolase I activity. *J. Biol. Chem.* 275:13275-13281.

26. Dassesse, D., Hemmens, B., Cuvelier, L., and Resibois, A. 1997. GTP-cyclohydrolase-I like immunoreactivity in rat brain. *Brain Res.* 777:187-201.

27. Messina, A., Knight, K.R., Dowsing, B.J., Zhang, B., Phan, L.H., Hurley, J.V., Morrison, W.A., and Stewart, A.G. 2000. Localization of inducible nitric oxide synthase to mast cells during ischemia/reperfusion injury of skeletal muscle. *Lab. Invest.* 80:423-431.

28. Niu, X.F., Ibbotson, G., and Kubes, P. 1996. A balance between nitric oxide and oxidants regulates mast cell- dependent neutrophil-endothelial cell interactions. *Circ. Res.* 79:992-999.

29. Werner-Felmayer, G., Werner, E.R., Fuchs, D., Hausen, A., Reibnegger, G., Schmidt, K., Weiss, G., and Wachter, H. 1993. Pteridine biosynthesis in human endothelial cells. Impact on nitric oxide-mediated formation of cyclic GMP. *J. Biol. Chem.* 268:1842-1846.

30. Sakai, N., Kaufman, S., and Milstein, S. 1993. Tetrahydrobiopterin is required for cytokine-induced nitric oxide production in a murine macrophage cell line (RAW 264). *Mol. Pharmacol.* 43:6-10.

31. Kasai, K., Hattori, Y., Banba, N., Hattori, S., Motohashi, S., Shimoda, S., Nakanishi, N., and Gross, S.S. 1997. Induction of tetrahydrobiopterin synthesis in rat cardiac myocytes: impact on cytokine-induced NO generation. *Am. J. Physiol.* 273:H665-H672.

32. Vasquez-Vivar, J. and Kalyanaraman, B. 2000. Generation of superoxide from nitric oxide synthase. *FEBS. Lett.* 481:305-306.

33. Brooks, A.C., Whelan, C.J., and Purcell, W.M. 1999. Reactive oxygen species generation and histamine release by activated mast cells: modulation by nitric oxide synthase inhibition. *Br. J. Pharmacol.* 128:585-590.

34. Wang, W., Wang, S., Yan, L., Madara, P., Del Pilar, C.A., Wesley, R.A., and Danner, R.L. 2000. Superoxide production and reactive oxygen species signaling by endothelial nitric-oxide synthase. *J. Biol. Chem.* 275:16899-16903.

35. Giordano, A., Tonello, C., Bulbarelli, A., Cozzi, V., Cinti, S., Carruba, M.O., and Nisoli, E. 2002. Evidence for a functional nitric oxide synthase system in brown adipocyte nucleus. *FEBS Lett.* 514:135-140.

36. Govers, R., van der, S.P., van Donselaar, E., Slot, J.W., and Rabelink, T.J. 2002. Endothelial nitric oxide synthase and its negative regulator caveolin-1 localize to distinct perinuclear organelles. *J. Histochem. Cytochem.* 50:779-788.

37. Sullivan, R., Burnham, M., Torok, K., and Koffer, A. 2000. Calmodulin regulates the disassembly of cortical F-actin in mast cells but is not required for secretion. *Cell Calcium* 28:33-46.

38. Kim, S.O., Merchant, K., Nudelman, R., Beyer, W.F., Jr., Keng, T., DeAngelo, J., Hausladen, A., and Stamler, J.S. 2002. OxyR: a molecular code for redox-related signaling. *Cell* 109:383-396.

Chapter 4

Expression, Localization and Regulation of Nitric Oxide Synthase (NOS) in Human Mast Cells: Effects on Leukotriene Production.

Authors, M. Gilchrist and A.D. Befus. At time of submission this chapter was under review at *Blood*. I contributed to this manuscript by designing and carrying out all the experiments and writing the manuscript.

Introduction

Mast cells (MC) are tissue-resident immune effector cells that arise from immature bone marrow precursors (1). They produce and secrete numerous bioactive agents and have been implicated in diverse homeostatic functions such as angiogenesis, wound healing and tissue remodeling (2). Due to their plethora of mediators and strategic localization, MC also play central roles in various disease states such as multiple sclerosis and T_H2-driven inflammatory conditions such as asthma (3,4).

Nitric oxide (NO) is a potent biological radical that plays diverse roles in regulating cellular activation (5). NO is derived from the guanidine group of L-arginine by the nitric oxide synthase (NOS) family of enzymes. The Ca^{2+}-dependent members include endothelial (eNOS) and neuronal (nNOS), characterized by constitutive expression and low NO production. Inducible NOS (iNOS) is upregulated by a variety of inflammatory mediators and functions independently of cellular Ca^{2+} levels and releases large amounts of NO (6). Numerous investigators have show that rodent MC are regulated by endogenous NO from both constitutive and inducible sources (7,8). However, little information is available concerning the production of NO by human MC, or the potential involvement of NO as a moderator in human MC populations.

The aim of the present study was therefore to investigate the expression of NOS and production of NO in human MC populations. Furthermore we studied the regulation of NOS, and the resulting functional effects on the release of leukotrienes. Our results indicate that NO may be a novel modulator in determining the functional phenotype of human MC.

Materials and Methods

Reagents

The NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), NO donor S-nitrosoglutathione (SNOG) were obtained from Calbiochem (San Diego, CA) and the NO probe 4,5-diaminofluorescein diacetate (DAF-FM) was obtained from Molecular Probes (Eugene, OR). Rabbit polyclonal antibodies against nNOS, iNOS and eNOS were obtained from Santa Cruz (Santa Cruz, CA) and

mouse monoclonals against the same proteins are from BD Transduction Laboratories (San Diego, CA). Rabbit polyclonal antibody against phosphorylated eNOS (Ser^{1177}) was obtained from Upstate Biotechnology (Lake Placid, NY) and rabbit anti-5-LO was obtained from Cayman Chemical (Ann Arbor, MI).

Cell lines

HMC-1, an immature human mast cell line (a kind gift from J.H. Butterfield, Minneapolis, MN), was cultured in Iscove's medium (Life Technologies, Grand Island, NY), with 10% fetal bovine serum (FBS), 2 mM glutamine, 40 U/mL penicillin/streptomycin and 1.0 mM thioglycerol. Cells were harvested and fed every 3-4 days. The human basophilic leukemia cells, KU812 (a kind gift from J.S. Marshall, Halifax, NS) were cultured in RPMI 1640 (Life Technologies) supplemented with 10% FBS, 2 nM glutamine, 10 mM HEPES and 40 U/mL penicillin/streptomycin. KU812 were differentiated by culture with 0.3 mM sodium butyrate for 3 days before use. The LAD 2 growth factor-dependent human mature mast cell line (obtained from Dr. A.S. Kirshenbaum and D.D. Metcalfe, NIH, Bethesda, MD) (9) were cultured in StemPro-34 medium (Life Technologies) supplemented with 100 ng/mL recombinant human stem cell factor (rhSCF)(Peprotech, Rocky Hill, NY). LAD 2 cells were split in culture every 7 d. All cells were maintained at 37°C in a humidified incubator at 5% CO_2.

Human skin mast cell isolation

Human skin MC (HSMC) were dispersed from 5 foreskin samples (mean age 2.4 ± 0.7 y, wet weight 0.7 ± 0.1 g) as previously described (10). Briefly, dissected samples were incubated for 1 hour at 37° C in collagenase (318 U/ml)(Life Technologies). Cells were separated through nylon gauze (150 μm) and were washed with complete RPMI 1640 (150g, 10 minutes). MC were enriched with 65% Percoll (Pharmacia Ltd.; Uppsala, Sweden). MC were further purified by positive magnetic selection using monoclonal anti-c-kit antibody (15 μg/mL, YB5.B8, BD Pharmingen) and goat anti-mouse Ig-coated magnetic beads (15 μg/mL, Miltenyi Biotec, Sunnyvale, CA). The average

yields were 4.5 ± 0.7 × 10^5 MC per foreskin sample with 93% ± 2.1% purity and 94% ± 1.3% viability assessed after staining with Kimura or trypan blue, respectively. The predominant contaminating cells were fibroblasts.

Sensitization of human mast cells

LAD 2 were sensitized with human IgE (2 μg/ml, Serotec) for 2 h at 37° C. Cells were washed and resuspended in HEPES-buffered Tyrode's solution, then stimulated with mouse anti-human IgE antibody (1 μg/mL, Serotec) for 30 minutes.

Reverse-transcriptase Polymerase Chain Reaction

Total RNA was isolated from MC using a modification of the Chomczynski/Sacchi method as previously described (11). One μg of RNA was converted to cDNA by the reverse transcription reaction (M.MLv reverse transcriptase, GIBCO/BRL) in a total volume of 20 μL. PCR amplification was performed on a PTC-100 Thermal Cycler (MJ Research, Boston, MA). The primers were designed to be intron-spanning based on published sequence data: nNOS, sense 5′-AAGGGCAATGTGCCTGTCGT-3′, antisense 5′-ATTGCCGTTGGCCTGAAGCA-3′ (PCR product, 826 bp); iNOS, sense 5′-AGTTTCTGGCAGCAACGG-3′, antisense 5′-TTAAGTTCTGTGCCGGCAG-3′ (PCR product, 532 bp); eNOS, sense 5′-ACCTGCAAAGCAGCAAGTCCACG-3′, antisense 5′-CCGAACACCAAAGTCATGGGAGT-3′ (PCR product, 837 bp); The number of cycles used for the primer sets was 25-35, (depending on the linear range). The conditions for PCR amplification were as follows: denaturing at 95°C for 45 s, annealing for 45 s, and extension at 72°C for 1 min. The optimized annealing temperature was 49°C for nNOS, 47°C for iNOS and 52°C for eNOS. Products were run on a 1.2 % agarose gel and stained with ethidium bromide (Sigma).

Western blot

MC were incubated in 24 well plates at 1 x 10^6 cells/well from 0 to 18 h in various experimental conditions. Cells were dissociated in RIPA buffer

(PBS,1% NP-40). The total protein content was determined by the Bradford technique (BioRad). Fifteen μg of protein from each sample was mixed with Lamelli loading buffer and separated on a 6% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (BioRad). The membrane was then incubated with primary antibodies for 1 h at room temperature. Dilutions of the primary antibodies are; polyclonal nNOS (2.5 μg/mL), iNOS (1.0 μg/mL), eNOS (0.5 μg/mL), phospho-eNOS (1.0 μg/mL) and 5-LO (5.0 μg/mL). The secondary antibody, HRP conjugated goat anti-rabbit IgG (0.05 μg/mL) (Serotec, Raleigh, NC) was added to the membrane and incubated for 1 h at room temperature. Labelling was detected by chemiluminescence by addition of SuperSignal substrate solution (Pierce, Rockford, IL). The resulting bands were scanned and quantified in a gel scanner (ImageMaster DTS, Pharmacia).

Subcellular fractionation

HMC-1 were fractionated as previously described (12). Briefly, cells were pelleted by centrifugation (150 g, 5 min) then resuspended in lysis buffer (10 mM HEPES, 2 mM $MgCl_2$, 15 mM KCl, 0.1 mM EDTA, and 0.2% Nonidet P-40, with protease inhibitors for 15 min on ice. Cells were ruptured with 12 passes of a Dounce homogenizer, then centrifuged at 10,000 g for 20 min. The supernatant was frozen as the cytoplasmic fraction. The pellet was resuspended in extraction buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 0.4 M NaCl, 20% glycerol, 0.2% EDTA and 0.2% NP-40 and incubated 20 min on ice, then centrifuged at 10,000 g for 20 min. The purity of nuclear extracts was tested by western blot using a β-tubulin antibody (Molecular Probes). All nuclear preps used in this study were negative for this cytoskeletal protein indicating minimal cytoplasmic contamination.

Confocal Microscopy

Localization of NOS in MC was performed on HMC-1 or LAD 2 fixed in 4% paraformaldehyde for 20 min, then permeablized with 0.1% triton X-100 in PBS. Slides were blocked for 30 min with 10% FBS and 3% BSA in PBS. All slides were incubated overnight at 4°C with primary antibody. Specific antibody binding was detected with rhodamine red-labeled goat anti-rabbit or

BODIPY- labeled mouse anti-rabbit secondary antibodies (Molecular Probes). Nuclei were stained with a 500 nM solution of propidium iodide (Molecular Probes). Negative controls with rabbit serum, were run concurrently. Cell images were obtained using a 40x 1.3 oil Plan-Neofluar objective on a Zeiss confocal laser scanning microscope (LSM510; Heidelberg, Germany). To determine nuclear colocalization FITC-stained images were combined with PI stained images of the same cell.

Assay for NOS activity

To categorize NO production in MC, NOS activity in HMC-1 was measured by the conversion of L-[^{14}C] arginine to L-[^{14}C] citrulline, using a NOS assay kit (Calbiochem, San Diego, CA) according to manufacturers procedures and as described previously (7). Each sample were run in the presence of EGTA (2mM) to determine the levels of calcium-dependent (constitutive) NOS activity. Calcium-independent (inducible) NOS activity was determined by subtracting the constitutive activity from the total NOS activity in the sample. The level of citrulline produced was expressed as picomoles/min/mg of protein. The protein content was determined by the Bradford technique (BioRad).

Diaminofluorescein assay for nitric oxide in human mast cells

NO production and localization in MC was assayed using DAF-FM, a cell-permeable NO-sensitive fluorescent dye as we have previously described (13,14). In brief, MC were loaded with 10 μM of DAF-FM for 1 h at 37°C, then pipetted onto coverslip-bottomed petri dishes (Falcon). Images were obtained using a Zeiss confocal laser scanning microscope. Images were collected using 488 nm (excitation) and 505-530 nm (emission) filter set, with a 40x 1.3 oil Plan-Neofluar objective. In some experiments the NOS inhibitor L-NAME, or the NO donor SNOG was added 30 minutes before loading with DAF as negative and positive controls respectively.

ELISA

HMC or LAD 2 (1 x 10^6/mL) were stimulated with A23187 (0.1 μM) or Anti-IgE (1 μg/mL) respectively, for 30 min at 37°C. Culture supernatants were

collected and cysteinyl leukotrienes quantified using EIA (Cayman Chemical) according to the manufacturers protocols.

Statistical analysis

All experiments were performed at least three times. Data was analyzed using analysis of varience (ANOVA) followed by the Bonferroni test for comparisons. *P* values < 0.01 were considered significant.

Results

NOS mRNA expression by human mast cells

Few studies have looked at NOS expression in human MC populations, and little firm data exists (15,16). Therefore we characterized the expression of nNOS, iNOS and eNOS mRNA in HMC-1, KU812, LAD 2 and human skin MC using RT-PCR analysis with specific primer pairs. As shown in Figure 4.1, at 30 cycles all human MC types studied expressed eNOS at high levels, while iNOS expression was negative in all samples even when cycle numbers were increased to 45. nNOS expression was variable, with KU812 and LAD 2 cells being negative, while HMC and HSMC were positive. However, nNOS levels were weaker when directly compared to eNOS at 30 cycles. NOS mRNA was detected with the appropriate controls; human brain RNA for nNOS and iNOS and human endothelial cell RNA for eNOS. The identity of all products was confirmed by cloning and sequencing. All PCR products were absent when the RT step was eliminated (data not shown).

NOS confocal

To determine if the expression of NOS mRNA could be correlated with the presence of NOS protein and to resolve the cellular localization patterns, laser scanning confocal microscopy was employed. Using this approach, HMC-1 stained weakly for nNOS protein with a diffuse pattern present throughout the cytoplasm (Fig. 4.2). LAD 2 cells and KU812 were negative for nNOS as expected from the PCR results (data not shown). Strikingly, HMC-1 stained with anti-eNOS antibody showed weak positive staining in the cytoplasm, whereas the nucleus showed very strong staining in a majority of cells. LAD 2 showed nuclear positivity in fewer cells (Fig. 4.2). iNOS protein was not

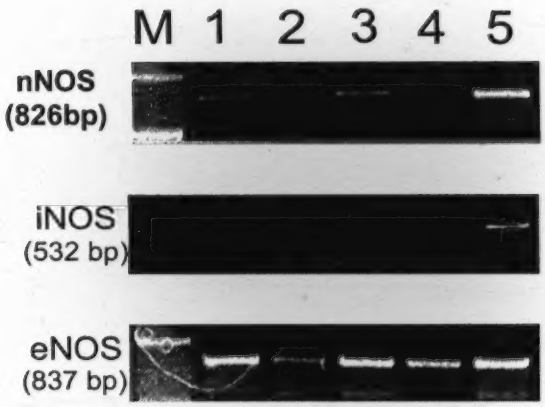


Fig. 4.1. Expression of nitric oxide synthase (NOS) isoforms in human mast cell (MC) populations. Neuronal NOS (nNOS): Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of nNOS messenger RNA (mRNA)in HMC-1 (lane 1), KU812 (lane 2), skin MC (lane 3), and LAD 2 (lane 4). Human brain RNA was used as a positive control (lane 5). Inducible NOS (iNOS): RT-PCR analysis of iNOS mRNA in HMC-1 (lane 1), KU812 (lane 2), skin MC (lane 3), and LAD 2 (lane 4). Human brain RNA was used as a positive control (lane 5). Endothelial NOS (eNOS): RT-PCR analysis of eNOS mRNA in HMC-1 (lane 1), KU812 (lane 2), skin MC (lane 3), and LAD 2 (lane 4). Human endothelial cell RNA was used as a positive control (lane 5). The sizes of expected amplified products are indicated (M). Results are representative of at least three independent RNA isolations.

detected in any cell type studied. There results were confirmed with a set of NOS monoclonal antibodies. Cells stained in parallel with non-immune rabbit serum were also negative.

As eNOS has been shown to localize to the golgi as well as to the peri-nuclear region in other cell types (17), colocalization in combination with the nuclear stain PI was used to confirm the nuclear eNOS staining. When the eNOS fluorescence signal was overlayed with the PI signal there was strong colocalization in the nucleus proper with some peripherally staining on the nuclear membrane as indicated by the yellow fluorescence in the merged image (Fig. 4.2). Interestingly, analysis of Z-stack section images taken every 2 μm throughout the cells showed that the localization pattern was not completely homogenous, with regions of high eNOS/DNA colocalization, interspersed with regions of almost no colocalization.

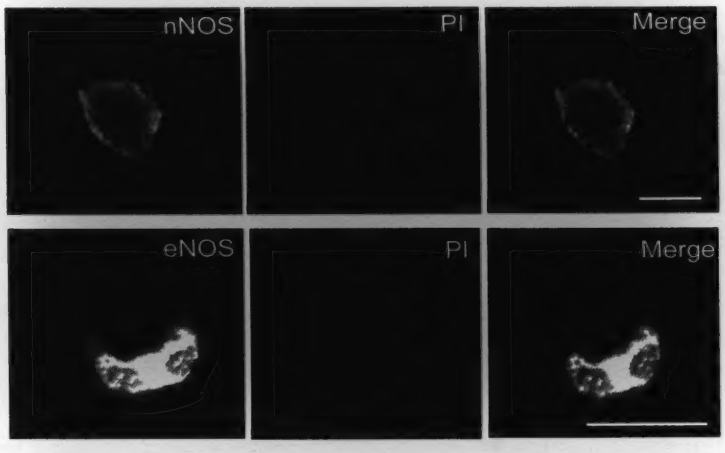
NOS protein

To further confirm the confocal results, and show that the observed localization patterns are not a result of staining artifacts, western blot analysis on purified nuclear and cytoplasmic extracts were investigated. HMC-1 cells contained cytoplasmic and nuclear eNOS, indicating that subcellular eNOS localization was unchanged from the confocal experiments (Fig.4.3). In addition, nNOS protein was found abundantly only in cytoplasmic fractions (Fig.4.3). Re-hybridization of the western blot with antibody against the cytoplasmic protein α-tubulin was used to validate the fractionation procedure (data not shown).

NOS activity and NO formation in human MC

We next investigated whether HMC-1 or LAD 2 cells were capable of NOS activity and NO formation. As NOS forms NO and L-citrulline from L-arginine in a stoichimetric reaction, we utilized the Citrulline assay to determine the conversion of radioactive L-arginine in HMC-1 homogenates. HMC-1 extracts showed significant citrulline generation (54.3±8.6 pmol/min/mg) that could be attributed to cNOS (nNOS or eNOS) but not iNOS activity, as chelation of Ca^{2+} from the reaction mixture completely abrogated citrulline formation (7.4±5.1 pmol/min/mg) (Fig. 4.4a). Interestingly, the levels of NOS activity in HMC-1

HMC-1



LAD 2

Fig. 4.2. Localization nitric oxide synthase (NOS) in HMC-1 and LAD 2 cells. HMC-1 or LAD 2 were fixed in 4% paraformaldehyde and incubated with rabbit anti-neuronal NOS (nNOS) antibody or rabbit anti-endothelial NOS (eNOS). Antibody labeling was detected with BODIPY-conjugated goat anti-rabbit antibodies (green). Nuclei of cells were detected with propidium iodide (red), overlapping fluorescence is indicated in yellow. Original magnification x 600, bar= 10 μm.

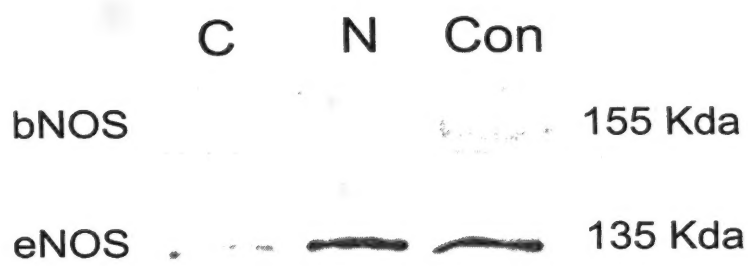


Fig. 4.3. Immunoblot analysis of nitric oxide synthase (NOS) protein expression in subcellular fractions from HMC-1. HMC-1 were lysed and separated into cytosolic (C) or nuclear (N) fractions as described under "Materials and Methods"; proteins from each fraction were separated by SDS-PAGE under reducing conditions. Control (Con) proteins (human brain and endothelial cell lysate respectively) were run concurrently. Representative immunoblot of neuronal NOS (nNOS) and endothelial NOS (eNOS) content in subcellular fractions. For all blots, equal amounts of protein (15 μg) were loaded. Molecular weight markers indicated at right. All blots are representative of at least three independent experiments.

were similar to that seen in brain homogenates (nNOS activity) used as a positive control (Fig.4.4a). These results further confirm that human MC are a potential source of NOS activity.

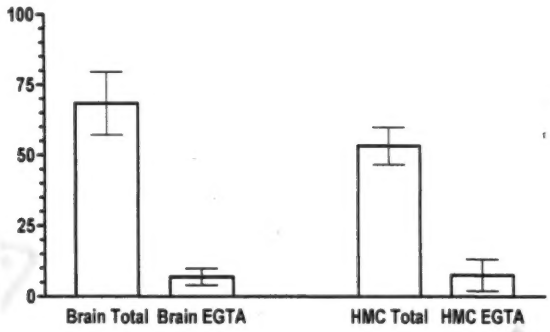
Because of the large number of LAD 2 cells that would be required for citrulline determinations and the slow growth rate of these cells in culture, combined with the fact that the citrulline assay does not directly measure NO production, we utililized the NO-specific fluorescent dye diaminofluorescein (DAF-2 DA) combined with live-cell confocal analysis to directly measure the dynamic production and localization of NO in both LAD 2 and HMC-1. Confocal images of DAF loaded HMC-1 showed minimal fluorescence. Upon stimulation with A23187 (1 μM), a majority (75±6.3 %, n=4) of HMC-1 showed a moderate increase in cytoplasmic fluorescence combined with pronounced nuclear localization beginning at 3 min with a plateau at 7 min (Fig. 4.4b). No significant increase in intracellular fluorescence was seen in time control experiments or if HMC-1 were pretreated with NOS inhibitor L-NAME (data not shown).

In parallel experiments preformed on IgE-sensitized LAD 2 cells we saw a similar pattern of NO formation following IgE crosslinking. Notably, the localization of NO was heterogenous, with some cells showing only cytoplasmic NO, while others showed both cytoplasmic and nuclear positivity (Fig. 4.4b). This data demonstrate that both HMC-1 and LAD 2 can produce endogenous NO upon stimulation.

Activation and co-distribution of eNOS and 5-LO

MC are critical effectors in inflammation due to their unique ability to differentially produce and release bioactive mediators such as leukotrienes (LT) (18). LT and NO are both formed in asthmatic inflammation, and interplay between the two pathways has been demonstrated (19,20). LT synthesis is initiated by the activation of 5-lipoxygenase (5-LO), which localizes to the nucleus in various MC populations (19). Since these proteins appear to reside in similar subcellular compartments we investigated eNOS and 5-LO distribution in MC with western blot.

A)



B)

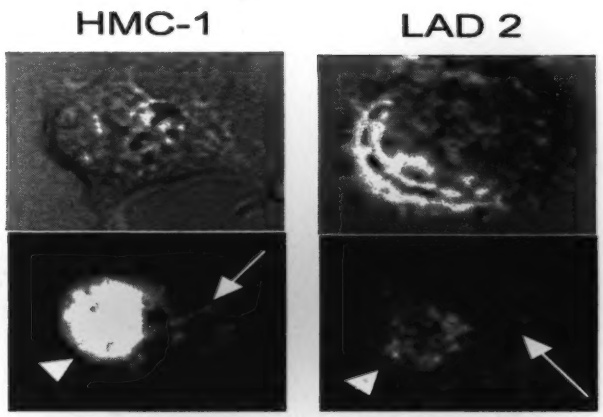


Fig. 4.4. Nitric oxide synthase (NOS) activity and nitric oxide (NO) localization in human mast cells. *A,* NOS activity in HMC-1, as measured using the [^{14}C]-L-arginine conversion assay. Brain homogenate was used as a positive control. Each sample were run in the presence of EGTA (2mM) to determine the levels of calcium-dependent (constitutive) NOS activity. The results are expressed as picomoles L-citrulline formed per minute, per mg of protein. Data shown as mean ± SEM for three independent experiments. *B,* Measurement of endogenous NO formation in HMC-1 and LAD 2 cells as detected by DAF fluorescence. Cells were loaded with diaminofluorescein (DAF), basal fluorescence images obtained, then stimulated with A23187 (1μM) or IgE-crosslinking respectively. Fluorescence changes were captured using confocal microscopy. White arrows show cytoplasmic DAF fluorescence, white arrowheads show nuclear/perinuclear positivity. Original magnification X 800. Results are representative of three independent experiments for HMC-1 and two for LAD 2.

As has been previously shown in rat MC (18), 5-LO in HMC-1 is mobilized predominantly nuclear compartments upon activation with A23197 (1 μM) in a time-dependent manner (Fig. 4.5a).

As constitutive NOS enzymes are also dependent on Ca^{2+} for full enzymatic activity, we investigated the effects of elevated intracellular Ca^{2+} on eNOS and nNOS localization in HMC-1. HMC-1 were treated with 1 μM A23187 for various time points prior to cell fractionation. There was a moderate change in eNOS localization with movement of the cytosolic eNOS into the nuclear fraction after A23187 treatment (2.5 fold increase) as determined by band densitometry (Fig. 4.5a). There was no appreciable change in nNOS localization (data not shown).

Previous studies showed that the presence of eNOS protein does not always correlate with NO production, as eNOS activity can be regulated by multiple post-translational modifications including phosphorylation (21). As phosphorylation at Ser^{1177} has been widely associated as an important mechanism for increasing NO formation (22), we measured phospho-eNOS (Ser^{1177}) levels in HMC-1 using specific antibodies. After 5 min of treatment with A23187 (1 μM), HMC-1 showed increased phospho-eNOS in both cytoplasmic (10 fold) and nuclear (3.5 fold) fractions compared with 0 time points. These data indicate that increased Ca^{2+} levels potentiate eNOS phosphorylation at Ser^{1177}, an indicator of increased eNOS activity (Fig. 4.5a). This increased activity correlates well with the time-course of DAF fluorescence seen in the confocal experiments.

Taken together with the confocal results, these data indicate that MC contain distinct cytoplasmic and nuclear pools of NOS and that eNOS can be mobilized and activated upon stimulation.

5-LO and eNOS colocalization

Given the similar expression pattern of 5-LO and eNOS we investigated the colocalization of these enzymes using confocal microscopy. In HMC-1, 5-LO colocalized with eNOS in both cytoplasmic and nuclear compartments, as shown by the similar patterns of anti-5LO (red) and anti-eNOS (green) staining

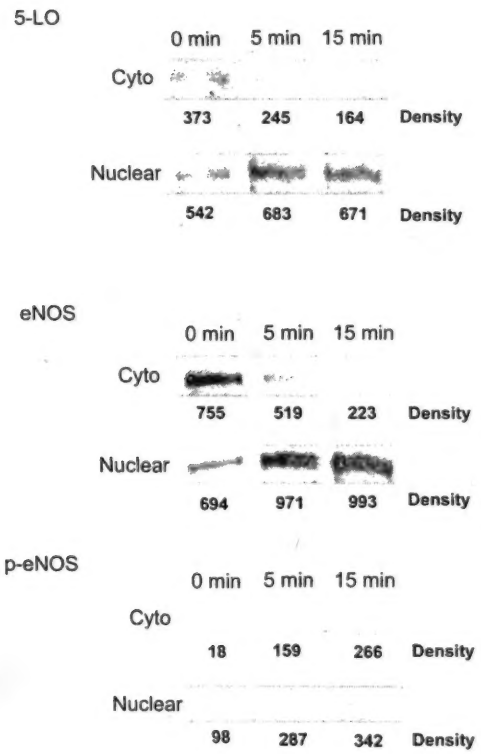


Fig. 4.5.A Translocation and colocalization of endothelial nitric oxide synthase (eNOS) and 5-lipoxygenase (5-LO) in HMC-1. *A,* Western blot analysis of 5-LO, eNOS and phospho-eNOS (Ser^{1177}) in subcellular fractions from HMC-1. Cells were stimulated with A23187 (1μM) for the indicated time points. Cell fractionation and western blotting were performed as described in "Materials and Methods". Equal quantities (15 μg) of protein were added to each lane, and the relative band intensities (numbers below the bands) were determined by densitometry (arbitrary units). Results are representative of three independent experiments.

Fig. 4.5.B. Translocation and colocalization of endothelial nitric oxide synthase (eNOS) and 5-lipoxygenase (5-LO) in HMC-1. *B,* Colocalization of eNOS and 5-LO in HMC-1. HMC-1 were fixed in 4% paraformaldehyde and incubated with rabbit anti-5-LO (red) and mouse anti-eNOS (green). Antibody labeling was detected with BODIPY-conjugated rabbit anti-mouse and rhodamine-red-conjugated goat anti-rabbit antibodies, overlapping fluorescence is indicated in yellow. Original magnification x 800, bar= 10 μm.

and confirmed by the yellow pattern in the merged overlays (Fig.4.5b). However, the colocalization is not complete, as noted by regions in both nucleus and cytoplasm that are free of colocalized signal. This data implies that 5-LO and eNOS protein colocalize, and may form a function regulatory unit based on this interaction.

Leukotriene ELISA/NO inhibitors

To confirm a biological role for NO in modulating human MC LT formation we analysed the release of LT in stimulated HMC-1 or LAD 2 supernatants after pretreatment with an NO donor (SNOG) or NOS inhibitor (L-NAME). Treatment of HMC-1 with A23187 (1 μM) (33.1±8.6 pg/mL, n=3) or crosslinking of IgE on LAD 2 (448±11.1 pg/mL, n=3) significantly increased cysteinyl leukotriene production over basal levels (HMC-1: 6.2±1.3 pg/mL, n=3; LAD 2: 4.6±2.8 pg/mL, n=3) in unstimulated cells (Fig. 4.6a&b). The levels of LT release were significantly lower in HMC-1 than in LAD 2 indicating important differences between immature and mature MC phenotypes. Pretreatment of cells with the NO donor SNOG for 30 min prior to activation caused a significant ($p<0.01$) and dose-dependent inhibition of short term LT release in both cell types (HMC-1: 51% , LAD-2: 63% inhibition)(Fig. 4.6a&b). Conversely, pretreatment with the NOS inhibitor L-NAME resulted in a significant ($p<0.01$) dose-dependent potentiation of LT release (HMC-1: 55%, LAD 2: 51%). Interestingly, although the amounts of LT release varied greatly between the two cell types, the NO donor and NOS inhibitor effected release to a similar extent. As NO can modulate cellular activities by activating soluble guanylate cyclase and increasing levels of cGMP (6) we examined the effect of a membrane permeant analogue of cGMP, 8-bromo-cGMP. 8-bromo-cGMP (1 mM) had no effect on A23187 or antigen driven LT release in either cell type (Fig. 4.6a&b).

Discussion

In rodents MC it has previously been shown that MC responsiveness and MC phenotype can be determined by NO (23). However there is little data concerning the production and effects of NO in human MC. Our results with

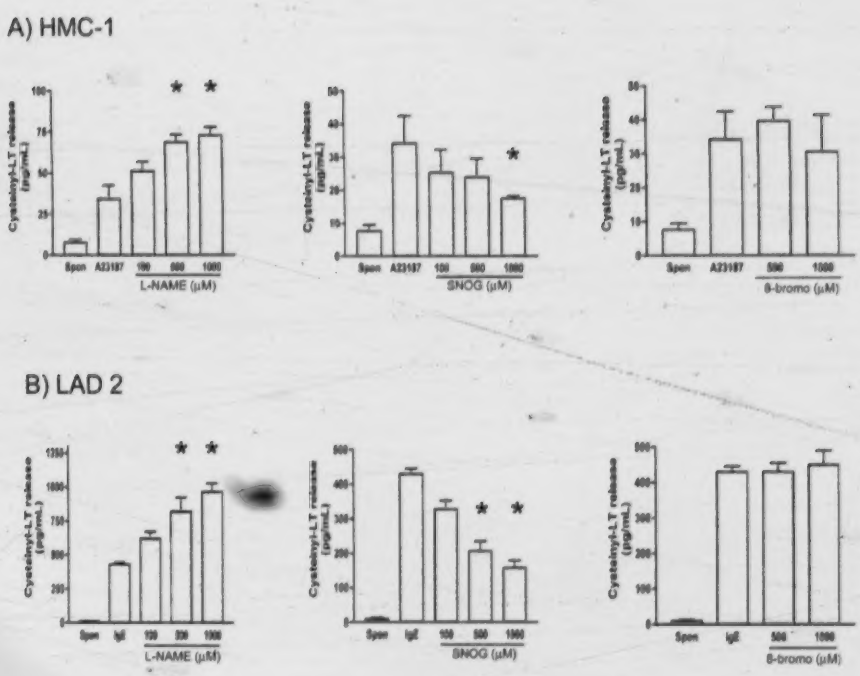


Fig. 4.6. Effect of nitric oxide synthase (NOS) inhibition and exogenous nitric oxide (NO) on cysteinyl leukotriene production in human mast cells (MC). *A,* Concentrations of cysteinyl leukotrienes in medium from HMC-1 was determined by ELISA. Cells were pre-incubation (30 min) with the NOS inhibitor (L-NAME), NO donor (SNOG) or cGMP analog (8-bromo-cGMP) then stimulated with A23187 (1 μM) for 30 min. Results are expressed as mean ± SEM for three independent experiments. *, Indicates $p < 0.01$ by comparison with untreated cells. *B,* Concentrations of cysteinyl leukotrienes in medium from LAD2 was determined by ELISA. Cells were pre-incubated (30 min) with the NOS inhibitor (L-NAME), NO donor (SNOG) or cGMP analog (8-bromo-cGMP) then stimulated with anti-IgE ((1 μg/mL) for 30 min. Results are expressed as mean ± SEM for three independent experiments. *, Indicates $p < 0.01$ by comparison with untreated cells.

show distinct differences in NOS expression and localization in a variety of human MC populations, with all MC types studied being positive for eNOS, but showing variable expression of nNOS, with no detectable iNOS.

Discerning distinct patterns is difficult, as both HMC-1 and KU812, while being immortalized cell lines, show differential expression of nNOS. The nature of the genetic phenotype of these cell lines may account for these differences, as HMC-1 have a mutation in the c-kit receptor (9). Furthermore, as HMC-1 are an immature MC line while KU812 are basophilic in phenotype may also account for this diversity. More detailed studies in blood basophils and other *in-vivo* MC populations may define these boundaries of NOS expression more clearly.

Comparison between the representative "mature" MC, namely HSMC and LAD-2, also show variable nNOS expression. Data on NOS expression in human MC is limited, but it has previously been shown by immunohistochemistry that both human skin and nasal mucosa MC are also positive for nNOS protein (16,24). Thus our nNOS results with *ex-vivo* HSMC are compatible with those *in-vivo* studies.

With the lack of iNOS expression, human MC studied thus far appear to rely on a unique expression profile based on constitutive (cNOS) in their quiescent state. This pattern is reiterated in other human immune cells, with neutrophils and eosinophils also being identified as cNOS expressors with variable expression of eNOS and nNOS isoforms (25,26). Interestingly, nNOS was also noted in eosinophils infiltrating dermal regions, implying that there may be specific environmental factors contributing to nNOS usage by immune cells in this particular cellular environment (26). Thus there are significant differences in NOS expression in human MC populations contributing further to MC heterogeneity.

The lack of iNOS expression is not surprising, given the "unstimulated" status of the MC populations studied and the need for appropriate stimuli for iNOS induction in other systems (6). It is interesting to note that during initial studies in several other human cell types including monocytes, macrophages, and

neutrophils there was also no iNOS expression detected, even when activated with known iNOS inducers such as IFN-γ and TNF (25,27). As studies have progressed iNOS expression has now been noted in all these cell types from patients with various disease states (28). However, the *in-vitro* conditions necessary to stimulate NO production remain incompletely characterized, and appropriate stimulation may also induce iNOS expression in human MC.

As little is also known about the distribution of NOS in human MC, the pattern of NOS localization was also noteworthy, with isoforms showing unique staining patterns. As eNOS has sites for lipid modification at is amino-terminal that can direct its binding, we expected plasma membrane localization as seen in endothelial cells (22,29). Interestingly, instead we saw diffuse cytoplasmic staining along with a very strong presence in the nucleus. Since other cell types have shown eNOS localization in the peri-nuclear or golgi regions (17) we confirmed nuclear eNOS by colocalization with PI in confocal analysis, or with subcellular fractionation and western blot. Also noteworthy was the translocation of eNOS from the cytoplasmic compartment to the nuclear fraction upon stimulation. Comparison to 5-LO, which is well known to redistribute to the MC nucleus upon activation (30), implies that these enzymes may share a common localization mechanism.

The possibility exists however that other, less-specific mechanisms, including non-specificity of the antibodies or antibody trapping in different cellular compartments may explain our localization results. Particularly in nuclear fractions where a plethora of charged proteins are present (31). To address these drawbacks we have used multiple techniques to determine the spatial localization of NOS proteins in human MC. Furthermore, we used multiple antibodies, from differing species, to confirm our confocal and western blot results, and utilized multiple fixation, permeablization and staining routines to also reconfirm our immunofluorescence results. Despite these precautions there are obvious limitations to the methodologies employed, and further investigations using transfection of fluorescent-tagged eNOS or 5-LO into MC,

or IP using purified proteins will help further confirm and define the distribution and interaction of these proteins.

Previous studies in other cell types have shown that eNOS protein can be detected in nuclear and peri-nuclear regions, and that eNOS may be active in such sites (17,22,32). Furthermore, studies have shown both Ca^{2+} and calmodulin accumulation at nuclear sites in activated MC (33,34), implicating that an environment may exist in the nucleus that is compliant to NO production. Defined roles for NO production in the nucleus are unknown, thus our data showing colocalization of eNOS with 5-LO and NO regulation of LT formation offer novel insights into potential mechansims. However, as NOS and 5-LO do not completely co-associate in the nucleus there are clearly other potential roles for each, including control of gene transcription by regulation of transcription factors (31).

The mechanisms involved in NO modulation of LT release from MC is likely complex. The best-known target for cellular NO is the heme moiety of the enzyme soluble guanylate cyclase, which increases cellular cGMP levels (6). Indeed, rapid signaling through eNOS-derived NO is modulated to a great extent by cGMP. However, our results with 8-bromo-cGMP showed no change in short-term LT release implying other mechanisms, which may include nitrosylation events. This data is consistent with that seen in other studies of the cGMP-independent effects of NO on MC secretion (degranulation) (35). Interestingly, these results were obtained using either NO donors, or iNOS driven cell systems to produce sustained NO to effect MC responses (36). Proposing that other NO/protein interactions (including nitrosylation) are responsible for the observed effects.

Indeed, NO derived from iNOS or NO donors has previously been shown to inhibit LT activity in macrophages through the direct nitrosylation of 5-LO (37). However, given the interaction of eNOS and 5-LO seen in our studies, such a mechanism may also be active in MC though through rapid eNOS activity compared to sustained iNOS activity. Indeed, it has been proposed that 5-LO exists in a "metabolon" of sequential enzymes in order that efficient production

of LT can proceed (19), and it is interesting to speculate that eNOS may be a regulatory component of this complex. Our western blot and confocal results show that eNOS and 5-LO appear to be regulated in a similar pattern with close association in cytoplasmic and nuclear domains. These results suggest that similar mechanisms may be employed in the trafficking and activation of these proteins within the cell.

The results from our study, and that of others, has shown that HMC-1 cells show weak and more variable LT release compared to more phenotypically mature MC (38). In fact, LT release in human MC populations is highly heterogeneous (39). As MC arise from a committed precursor that mature in distinct environments, MC variability is thus greatly modified by environmental factors (1). Indeed, LAD 2 cells showed less DAF (NO) positive cells than HMC-1 upon activation, and released significantly more LT. It is interesting to speculate that these environmental influences may also drive NOS expression, including increase nNOS production, with varying effects of tonic NO production on MC function and mediator release.

Much previous research has centered on the role played by iNOS in inflammation and the effects caused by the high levels of NO produced (6). More recently, however, there has been an evolving role of eNOS in immune cell regulation. Indeed, using NOS knockout mice a vital role for eNOS in propagating immune processes in macrophages has been identified (40). Further knockout studies have identified an important function for eNOS in initiating leukocyte influx into inflammatory sites (41). In addition, an unexpected role for nNOS, not iNOS, was identified in mouse models of asthmatic inflammation (42). Our studies further expand this understanding, and indicate that there are clear cell specific differences in the roles played by cNOS. Unfortunately our results cannot exclusively dissect discreet roles for eNOS or nNOS in cells expressing both isoforms and investigations with knockout/knockdown strategies will help delineate the role of cNOS in inflammation.

The hallmarks of the MC are its armamentarium of granule-stored mediators, de-novo lipid derived molecules and newly-formed cytokines that can be differentially and rapidly released according to the stimulus (43). Such a complex interplay must arise from an involved and necessarily overlapping control machinery (43). Previous studies in rodent MC (7,36), along with more recent studies in human basophils (44), have shown a role for both endogenous and exogenous NO inhibition of granule mediator secretion. We have shown here for the first time that MC-derived NO modulates LT production formed immediately (< 30 min) after activation. In addition, we have shown in this and previous studies (13), NO formation and NOS localization within the nucleus which may account for the NO regulation of chemokine and cytokine production seen in other cell types (45). NO thus appears to be strategically placed to potentially impinge on all of these pathways due the differential localization of NOS with the capacity for intense, directed production of this potent radical.

References

1. Gurish, M.F. and Austen, K.F. 2001. The diverse roles of mast cells. *J. Exp. Med.* 194:F1-F5.

2. Wedemeyer, J., Tsai, M., and Galli, S.J. 2000. Roles of mast cells and basophils in innate and acquired immunity. *Curr. Opin. Immunol.* 12:624-631.

3. Benoist, C. and Mathis, D. 2002. Mast cells in autoimmune disease. *Nature* 420:875-878.

4. Brightling, C.E., Bradding, P., Pavord, I.D., and Wardlaw, A.J. 2003. New insights into the role of the mast cell in asthma. *Clin. Exp. Allergy* 33:550-556.

5. Grisham, M.B., Jourd'Heuil, D., and Wink, D.A. 1999. Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites:implications in inflammation. *Am. J. Physiol.* 276:G315-G321.

6. Alderton, W.K., Cooper, C.E., and Knowles, R.G. 2001. Nitric oxide synthases: structure, function and inhibition. *Biochem. J.* 357:593-615.

7. Gilchrist, M., Savoie, M., Nohara, O., Wills, F.L., Wallace, J.L., and Befus, A.D. 2002. Nitric oxide synthase and nitric oxide production in in vivo-derived mast cells. *J. Leukoc. Biol.* 71:618-624.

8. Kashiwagi, S., Kajimura, M., Yoshimura, Y., and Suematsu, M. 2002. Nonendothelial source of nitric oxide in arterioles but not in venules: alternative source revealed in vivo by diaminofluorescein microfluorography. *Circ. Res.* 91:e55-e64.

9. Kirshenbaum, A.S., Akin, C., Wu, Y., Rottem, M., Goff, J.P., Beaven, M.A., Rao, V.K., and Metcalfe, D.D. 2003. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. *Leuk. Res.* 27:677-682.

10. Willheim, M., Agis, H., Sperr, W.R., Koller, M., Bankl, H.C., Kiener, H., Fritsch, G., Fureder, W., Spittler, A., and Graninger, W. 1995. Purification of human basophils and mast cells by multistep separation technique and mAb to CDw17 and CD117/c-kit. *J. Immunol. Methods* 182:115-129.

11. Gilchrist, M., MacDonald, A.J., Neverova, I., Ritchie, B., and Befus, A.D. 1997. Optimization of the isolation and effective use of mRNA from rat mast cells. *J. Immunol. Methods* 201:207-214.

12. Mohamed, A.J., Vargas, L., Nore, B.F., Backesjo, C.M., Christensson, B., and Smith, C.I. 2000. Nucleocytoplasmic shuttling of Bruton's tyrosine kinase. *J. Biol. Chem.* 275:40614-40619.

13. Gilchrist, M., Hesslinger, C., and Befus, A.D. 2003. Tetrahydrobiopterin: Critical factor in the production and role of nitric oxide in mast cells. *J. Biol. Chem.* In press.

14. Suzuki, N., Kojima, H., Urano, Y., Kikuchi, K., Hirata, Y., and Nagano, T. 2002. Orthogonality of calcium concentration and ability of 4,5-diaminofluorescein to detect NO. *J. Biol. Chem.* 277:47-49.

15. Bidri, M., Feger, F., Varadaradjalou, S., Ben Hamouda, N., Guillosson, J.J., and Arock, M. 2001. Mast cells as a source and target for nitric oxide. *Int. Immunopharmacol.* 1:1543-1558.

16. Bacci, S., Arbi-Riccardi, R., Mayer, B., Rumio, C., and Borghi-Cirri, M.B. 1994. Localization of nitric oxide synthase immunoreactivity in mast cells of human nasal mucosa. *Histochemistry* 102:89-92.

17. Govers, R., van der, S.P., van Donselaar, E., Slot, J.W., and Rabelink, T.J. 2002. Endothelial nitric oxide synthase and its negative regulator caveolin-1 localize to distinct perinuclear organelles. *J. Histochem. Cytochem.* 50:779-788.

18. Brock, T.G., McNish, R.W., and Peters-Golden, M. 1995. Translocation and leukotriene synthetic capacity of nuclear 5-lipoxygenase in rat basophilic leukemia cells and alveolar macrophages. *J. Biol. Chem.* 270:21652-21658.

19. Peters-Golden, M. and Brock, T.G. 2001. Intracellular compartmentalization of leukotriene synthesis: unexpected nuclear secrets. *FEBS Lett.* 487:323-326.

20. Coffey, M.J., Phare, S.M., and Peters-Golden, M. 2002. Interaction between nitric oxide, reactive oxygen intermediates, and peroxynitrite in the regulation of 5-lipoxygenase metabolism. *Biochim. Biophys. Acta.* 1584:81-90.

21. Fleming, I. and Busse, R. 2003. Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 284:R1-12.

22. Fulton, D., Fontana, J., Sowa, G., Gratton, J.P., Lin, M., Li, K.X., Michell, B., Kemp, B.E., Rodman, D., and Sessa, W.C. 2002. Localization of endothelial nitric-oxide synthase phosphorylated on serine 1179 and nitric oxide in Golgi and plasma membrane defines the existence of two pools of active enzyme. *J. Biol. Chem.* 277:4277-4284.

23. Coleman, J.W. 2002. Nitric oxide: a regulator of mast cell activation and mast cell- mediated inflammation. *Clin. Exp. Immunol.* 129:4-10.

24. Shimizu, Y., Sakai, M., Umemura, Y., and Ueda, H. 1997. Immunohistochemical localization of nitric oxide synthase in normal human skin: expression of endothelial-type and inducible-type nitric oxide synthase in keratinocytes. *J. Dermatol.* 24:80-87.

25. Greenberg, S.S., Ouyang, J., Zhao, X., and Giles, T.D. 1998. Human and rat neutrophils constitutively express neural nitric oxide synthase mRNA. *Nitric. Oxide.* 2:203-212.

26. Maruo, K., Kayashima, K.I., and Ono, T. 1999. Expression of neuronal nitric oxide synthase in dermal infiltrated eosinophils in eosinophilic pustular folliculitis. *Br. J. Dermatol.* 140:417-420.

27. Weinberg, J.B., Misukonis, M.A., Shami, P.J., Mason, S.N., Sauls, D.L., Dittman, W.A., Wood, E.R., Smith, G.K., McDonald, B., and Bachus, K.E. 1995. Human mononuclear phagocyte inducible nitric oxide synthase (iNOS): analysis of iNOS mRNA, iNOS protein, biopterin, and nitric oxide production by blood monocytes and peritoneal macrophages. *Blood* 86:1184-1195.

28. Fang, F.C. and Vazquez-Torres, A. 2002. Nitric oxide production by human macrophages: there's NO doubt about it. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 282:L941-L943.

29. Albrecht, E.W., Stegeman, C.A., Heeringa, P., Henning, R.H., and van Goor, H. 2003. Protective role of endothelial nitric oxide synthase. *J. Pathol.* 199:8-17.

30. Hanaka, H., Shimizu, T., and Izumi, T. 2002. Nuclear-localization-signal-dependent and nuclear-export-signal-dependent mechanisms determine the localization of 5-lipoxygenase. *Biochem. J.* 361:505-514.

31. Haqqani, A.S., Kelly, J.F., and Birnboim, H.C. 2002. Selective nitration of histone tyrosine residues in vivo in mutatect tumors. *J. Biol. Chem.* 277:3614-3621.

32. Giordano, A., Tonello, C., Bulbarelli, A., Cozzi, V., Cinti, S., Carruba, M.O., and Nisoli, E. 2002. Evidence for a functional nitric oxide synthase system in brown adipocyte nucleus. *FEBS Lett.* 514:135-140.

33. Mori, S., Saino, T., and Satoh, Y. 2000. Effect of low temperatures on compound 48/80-induced intracellular Ca2+ changes and exocytosis of rat peritoneal mast cells. *Arch. Histol. Cytol.* 63:261-270.

34. Sullivan, R., Burnham, M., Torok, K., and Koffer, A. 2000. Calmodulin regulates the disassembly of cortical F-actin in mast cells but is not required for secretion. *Cell Calcium* 28:33-46.

35. Deschoolmeester, M.L., Eastmond, N.C., Dearman, R.J., Kimber, I., Basketter, D.A., and Coleman, J.W. 1999. Reciprocal effects of interleukin-4 and interferon-gamma on immunoglobulin E-mediated mast cell degranulation: a role for nitric oxide but not peroxynitrite or cyclic guanosine monophosphate. *Immunology* 96:138-144.

36. Eastmond, N.C., Banks, E.M., and Coleman, J.W. 1997. Nitric oxide inhibits IgE-mediated degranulation of mast cells and is the principal intermediate in IFN-gamma-induced suppression of exocytosis. *J. Immunol.* 159:1444-1450.

37. Coffey, M.J., Phare, S.M., and Peters-Golden, M. 2000. Prolonged exposure to lipopolysaccharide inhibits macrophage 5-lipoxygenase metabolism via induction of nitric oxide synthesis. *J. Immunol.* 165:3592-3598.

38. Macchia, L., Hamberg, M., Kumlin, M., Butterfield, J.H., and Haeggstrom, J.Z. 1995. Arachidonic acid metabolism in the human mast cell line HMC-1: 5-lipoxygenase gene expression and biosynthesis of thromboxane. *Biochim. Biophys. Acta.* 1257:58-74.

39. Hsieh, F.H., Lam, B.K., Penrose, J.F., Austen, K.F., and Boyce, J.A. 2001. T helper cell type 2 cytokines coordinately regulate immunoglobulin E-dependent cysteinyl leukotriene production by human cord blood-derived mast cells: profound induction of leukotriene C(4) synthase expression by interleukin 4. *J. Exp. Med.* 193:123-133.

40. Connelly, L., Jacobs, A.T., Palacios-Callender, M., Moncada, S., and Hobbs, A.J. 2003. Macrophage endothelial nitric-oxide synthase autoregulates cellular activation and pro-inflammatory protein expression. *J. Biol. Chem.* 278:26480-26487.

41. Lefer, D.J., Jones, S.P., Girod, W.G., Baines, A., Grisham, M.B., Cockrell, A.S., Huang, P.L., and Scalia, R. 1999. Leukocyte-endothelial cell interactions in nitric oxide synthase-deficient mice. *Am. J. Physiol.* 276:H1943-H1950.

42. De Sanctis, G.T., MacLean, J.A., Hamada, K., Mehta, S., Scott, J.A., Jiao, A., Yandava, C.N., Kobzik, L., Wolyniec, W.W., Fabian, A.J., Venugopal, C.S., Grasemann, H., Huang, P.L., and Drazen, J.M. 1999. Contribution of nitric oxide synthases 1, 2, and 3 to airway hyperresponsiveness and inflammation in a murine model of asthma. *J. Exp. Med.* 189:1621-1630.

43. Kawakami, T. and Galli, S.J. 2002. Regulation of mast-cell and basophil function and survival by IgE. *Nat. Rev. Immunol* 2:773-786.

44. Iikura, M., Takaishi, T., Hirai, K., Yamada, H., Iida, M., Koshino, T., and Morita, Y. 1998. Exogenous nitric oxide regulates the degranulation of human basophils and rat peritoneal mast cells. *Int. Arch. Allergy Immunol.* 115:129-136.

45. Clancy, R., Varenika, B., Huang, W., Ballou, L., Attur, M., Amin, A.R., and Abramson, S.B. 2000. Nitric oxide synthase/COX cross-talk: nitric oxide activates COX-1 but inhibits COX-2-derived prostaglandin production. *J. Immunol.* 165:1582-1587.

Chapter 5

IFN-γ Regulates Chemokine Expression and Release in Human Mast Cells: Role of Nitric Oxide

Introduction

Mast cells (MC) are bone marrow-derived, tissue-resident effector cells (1). They produce and secrete a variety of preformed and newly-synthesized mediator that have diverse roles in homeostasis and disease (2). Due to their diversity of mediators and strategic localization, MC have been implicated in disease states such as multiple sclerosis and inflammatory conditions such as asthma (3,4).

The archetypal T_H1 cytokine, interferon-γ (IFN-γ), has been shown in numerous systems to down-regulate various MC responses (5,6). Previous studies have shown that IFN-γ inhibits MC adhesion as well as modulating MC mediator secretion (7,8). The role that IFN-γ plays in regulating human MC is less defined, though recent studies have shown that IFN-γ may be important in sculpting MC phenotype by determining a distinct pattern of cytokine/chemokine expression (9). Interestingly, down-regulation of IFN-γ levels may be a contributing factor in human asthma (10).

One of the many effects down-stream of IFN-γ is the production of the gaseous radical, nitric oxide (NO)(6). NO is derived from the guanidine group of L-arginine by the nitric oxide synthase (NOS) family of enzymes. The Ca^{2+}-dependent members include endothelial (eNOS) and neuronal (nNOS), characterized by constitutive expression and low NO production. Inducible NOS (iNOS) is upregulated by a variety of inflammatory mediators and functions independently of cellular Ca^{2+} levels and releases large amounts of NO (11). Numerous investigators have show that rodent MC are regulated by endogenous NO from both constitutive and inducible sources (12,13), and it has been shown that IFN-γ regulation of MC mediator secretion and adhesion in rodents is NO-dependent (7,8). We have recently shown that human MC populations are capable of nNOS and eNOS expression and NO-dependent regulation of leukotriene release (14). However, little information is available concerning the potential involvement of NO as a moderator of IFN-γ effects in human MC populations.

The aim of the present study was therefore to investigate the regulation of NOS by IFN-γ, and the resulting functional effects on the expression and release of the chemokines; monocyte chemoattractant protein-1 (MCP-1) and I-309. These

results indicate that NO may be a novel modulator in determining the functional phenotype of human MC.

Materials and Methods

Reagents

The NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) and NO donor S-nitrosoglutathione (SNOG) were obtained from Calbiochem (San Diego, CA). Rabbit polyclonal antibodies against nNOS, iNOS and eNOS were obtained from Santa Cruz (Santa Cruz, CA).

Cell lines

HMC-1, an immature human mast cell line (a kind gift from J.H. Butterfield, Minneapolis, MN), was cultured in Iscove's medium (Life Technologies, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 40 U/mL penicillin/streptomycin and 1.0 mM thioglycerol. Cells were harvested and fed every 3-4 days and maintained at 37°C in a humidified incubator at 5% CO_2.

Gene array

HMC-1 were treated with IFN-γ (200 U/mL) (R&D Systems, Minneapolis, MN) for 18 h. Total RNA was isolated using a modification of the Chomczynski/Sacchi method as previously described (15) and compared against unstimualted HMC-1 by the use of cDNA expression array specific for human sequences (R&D Systems). RNA (5 μg) was used as the template for ^{32}P dCTP-labeled probe synthesis. After hybridization for 18 h the array was washed then exposed to X-ray film (Kodak) for 48 h at -80°C. The spot intensities were quantified by densitometry (SigmaGel, SPSS Science, Chicago, IL). Expression levels were measured as signal/background (negative control) ratio with a 2 fold-difference between populations considered as differentially regulated as previously described (16).

Semi-quantitative Reverse-transcriptase Polymerase Chain Reaction

Semi-quantitative RT-PCR was run as we have previously described (12). One μg of RNA was converted to cDNA by the reverse transcription reaction (M.MLv reverse transcriptase, GIBCO/BRL) in a total volume of 20 μL. PCR amplification was performed on a PTC-100 Thermal Cycler (MJ Research, Boston, MA). The

primers were designed to be intron-spanning based on published sequence data: nNOS, sense 5′-AAGGGCAATGTGCCTGTCGT-3′, antisense 5′-ATTGCCGTTGGCCTGAAGCA-3′ (PCR product, 826 bp); iNOS, sense 5′-AGTTTCTGGCAGCAACGG-3′, antisense 5′-TTAAGTTCTGTGCCGGCAG-3′ (PCR product, 532 bp); eNOS, sense 5′-ACCTGCAAAGCAGCAAGTCCACG-3′, antisense 5′-CCGAACACCAAAGTCAT GGGAGT-3′ (PCR product, 837 bp); MCP-1, sense 5′-CAGAAGTGGGTTCAGGATTC-3′, antisense 5′-GGGTAGAACTGTGGTTCAAG-3′ (PCR product, 379 bp); I-309, sense 5′-GACCAGAAGACATGCAGATC-3′, antisense 5′-TCCAGAGCCCACAATGGAAA-3′ (PCR product, 330 bp); β-actin, sense 5′-GTGGGGCGCCCCAGGCACCA-3′, antisense 5′-GTCCTTAATGTCACGCACGATTT

C- 3′ (PCR product, 526 bp). The number of cycles used for the primer sets was 25-35, (depending on the linear range). The conditions for PCR amplification were as follows: denaturing at 95°C for 45 s, annealing for 45 s, and extension at 72°C for 1 min. The optimized annealing temperature was 49°C for nNOS, 47°C for iNOS, I-309, MCP-1, 52°C for eNOS and 56°C. For semi-quantitative PCR analysis, during amplification aliquots were withdrawn at 26, 29, 32 and 35 cycles to insure that amplification was within the exponential range. Products were run on a 1.2 % agarose gel and stained with ethidium bromide (Sigma). Band intensities were obtained with a gel scanner (Imagemaster, Pharmacia, Denver, CO).

Western blot

HMC-1 were incubated in 24 well plates at 1 x 10^6 cells/well from 0 to 18 h in various experimental conditions. The cells were dissociated by 500 μL of RIPA buffer (PBS,1% NP-40). The total protein content of each sample was determined by the Bradford technique (BioRad). Fifteen μg of protein from each sample was mixed with Lamelli loading buffer containing SDS and β-mercaptoethanol. Samples were electrophoretically separated on a 12% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (BioRad). The membrane was then incubated with primary antibodies for 1 h at room temperature. Dilutions

of the primary antibodies are; polyclonal nNOS (2.5 μg/mL), iNOS (1.0 μg/mL) and eNOS (0.5 μg/mL). The secondary antibody, HRP conjugated goat anti-rabbit IgG (0.05 μg/mL)(Serotec, Raleigh, NC) was added to the membrane and incubated for 1 h at room temperature. Labelling was detected by chemiluminescence by addition of SuperSignal substrate solution (Pierce, Rockford, IL).

Assay for NOS activity

To further categorize NO production in MC, NOS activity was measured by the conversion of L-[^{14}C] arginine to L-[^{14}C] citrulline, using a NOS assay kit (Calbiochem) according to manufacturers procedures and as we have previously published (12). Duplicate incubations at 37 ^{o}C for each sample were run for 30 min in the presence of EGTA (2mM) to determine the levels of calcium-dependent (constitutive) NOS activity. Calcium-independent (inducible) NOS activity was determined by subtracting the constitutive activity from the total NOS activity in the sample. The level of citrulline produced was expressed as picomoles/min/mg of protein. The protein content was determined by the Bradford technique (BioRad). Blank values were obtained from boiled cell extracts were subtracted from all test samples. Specificity of the assay was validated by adding the pan-NOS inhibitor, NG -monomethyl- L-arginine (L-NMMA) (100 μM), to ensure that citrulline detected arose from the activity of NOS.

ELISA for chemokines

MCP-1 and I-309 release was measured in MC culture supernatants 4 h after stimulation using specific commercially available ELISA according to the manufacturers protocols. Sensitivity of the MCP-1 assay is 5.0 pg/mL and that of I-309 is 0.01 pg/mL.

Statistical analysis

All experiments were performed at least three times. Data was analyzed using analysis of varience (ANOVA) followed by the Bonferroni test for comparisons. *P* values < 0.05 were considered significant.

Results

Identification of IFN-γ regulated gene expression in HMC-1 by cDNA array

IFN-γ is a well-known regulator of gene expression (17). To search for genes regulated by IFN-γ in human MC we performed cDNA expression array analysis in HMC-1. Of the 387 genes present on the array, HMC-1 RNA hybridized with 60 (60/387=15.5%) with IFN-γ upregulating seven (7/60=12%), downregulating 21 (21/60=35%) and 32 unchanged (32/60=53%). Table 5.I shows a crosssection of the array results. Notworthy was the upregulation of eNOS (+4.7 fold) and downregulation of the chemokines MCP-1 (-5.2 fold) and I-309 (-3.6 fold).

Confirmation of IFN-γ regulation of NOS mRNA expression

As there can be a moderate error rate with gene array analysis combined with lower sensitivity we looked at NOS isoform mRNA regulation by IFN-γ with RT-PCR to confirm the array results. Total RNA was extracted from unstimulated HMC-1, and from HMC-1 treated with IFN-γ (200 U/mL) for 6 and 18 h. NOS mRNA production was assessed by semiquantitative RT-PCR vs β-actin (housekeeping gene)(Fig. 5.1A). Within 6 h following treatment with IFN-γ both the nNOS and eNOS signal in HMC-1 increased with eNOS significantly increasing ($p<0.05$) almost 4 fold. Levels of eNOS mRNA reached maximal expression at 6 h and leveled off at 18 h (Fig. 5.1A&B). No change in NOS mRNA was noted in unstimulated HMC-1 at similar time points (data not shown). Furthermore, iNOS mRNA was not detected. Results are from RNA obtained from three independent batches of HMC-1.

NOS protein expression

To evaluate the effects of IFN-γ on NOS protein expression, Western blot analysis was employed. Unstimulated HMC-1 constitutively produced nNOS (155 kDa) and eNOS (135 kDa)(Fig. 5.2). Treatment with IFN-γ (200 U/mL) produced an increase in eNOS protein expression, with maximal stimulation at 18 h. There was a little increase in nNOS expression (Fig. 5.2). Untreated HMC-1 cultured during the same time showed no increase in NOS protein expression, nor was iNOS protein detected in any of the experiments (data not shown).

Table 5.I. Genes significantly regulated by interferon-γ (IFN-γ) in HMC-1.

Gene Name	Accession Number	Differential Expression
c-kit	X06182	+1.2
Urokinase receptor	Z46797	-4.9
NGF-R	M14764	-2.1
MCP-1	S69738	-5.2
I-309	M57502	-3.6
IK	S74221	-1.7
MIF	M25639	-1.3
eNOS	L26914	+4.7

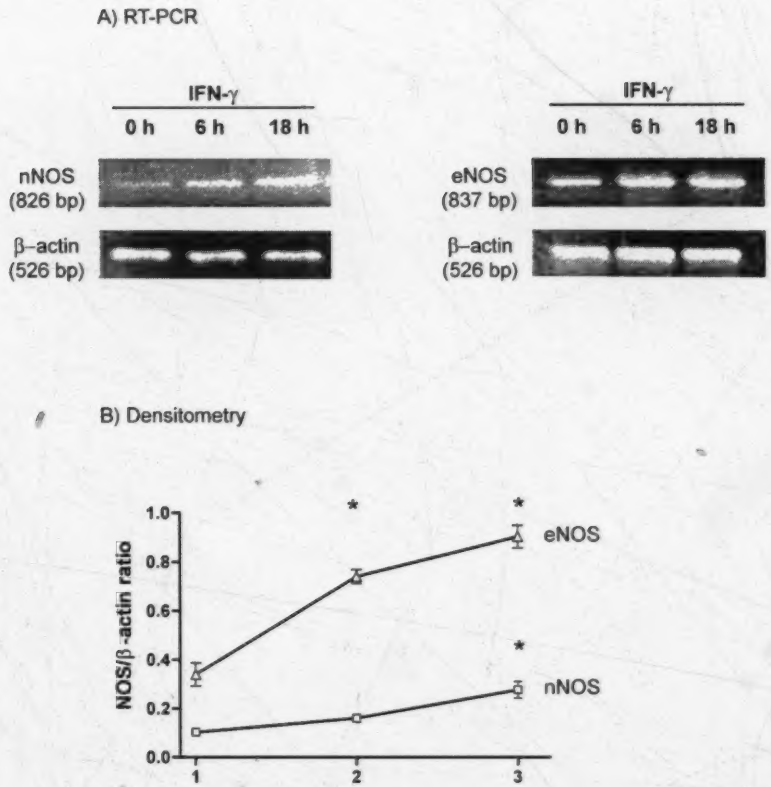


Fig. 5.1. Interferon-γ (IFN-γ) regulation of nitric oxide synthase (NOS) messenger (RNA (mRNA) expression in HMC-1. *A,* mRNA levels for neuronal NOS (nNOS), endothelial NOS (eNOS) and β–actin by RT-PCR in HMC-1 cultured with 800U/mL IFN-γ for 0 (1), 6 (2) and 18 (3) h. *B,* Densitometric analyses are shown as the NOS/β-actin ratio, expressed in arbitrary units. The PCR gels shown are representative of two others performed with different batches of HMC-1 RNA. Error bars represent SEM from three separate experiments. *, indicates $p<0.05$ compared to unstimulated cells.

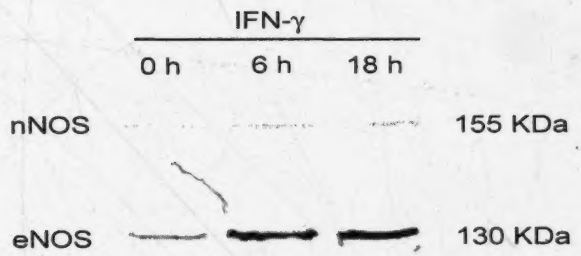


Fig. 5.2. Interferon-γ (IFN-γ) regulation of nitric oxide synthase (NOS) protein expression in HMC-1. Western blot analysis using anti-neuronal NOS (nNOS) and endothelial NOS (eNOS) antibody with 15 μg of total cell homogenate obtained from HMC-1 treated with IFN-γ (200 U/mL) for the indicated times. Results are representative of three separate experiments.

NOS activity and NO formation in HMC-1

We next investigated NOS activity in HMC-1 homogenates using the citrulline assay. As we have previously shown (14), HMC-1 extracts showed significant citrulline generation (54.3±6.0 pmol/min/mg) that could be attributed to cNOS (nNOS or eNOS) but not iNOS activity, as chelation of Ca^{2+} from the reaction mixture abrogated citrulline formation (7.4±5.1 pmol/min/mg)(Fig. 5.3). HMC-1 treated for 18 h with IFN-γ (200 U/mL) showed a significant ($p<0.05$) upregulation of cNOS activity (70.1±6.9 pmol/min/mg)(Fig. 5.3). Rat brain homogenates were run concurrently as a positive control. These results further confirm that human MC are a potential source of NOS activity.

Regulation of MCP-1 and I-309 mRNA expression by NO

To confirm the gene array results showing inhibition of MCP-1 and I-309 mRNA expression we performed RT-PCR analysis with total RNA isolated from IFN-γ (200 U/mL) treated HMC-1. As shown in Fig. 5.4, both MCP-1 and I-309 mRNA were significantly ($p<0.05$) inhibited by IFN-γ (52% and 34 %, respectively). To determine if NO plays an indirect role in the IFN-γ modulation of HMC-1 chemokine expression, HMC-1 were pretreated (18 h) with the NOS inhibitor, L-NAME (100 μM), or the NO donor, SNOG (100 μM) in combination with IFN-γ (200 U/mL). Total RNA was isolated and analyzed for MCP-1 and I-309 expression (Fig. 5.4 A&B). HMC-1 treated with both IFN-γ and L-NAME removed the inhibitory effect of IFN-γ with both MCP-1 and I-309. Interestingly, addition of the NO donor, SNOG, to IFN-γ treated cells potentiated the inhibition of MCP-1 mRNA expression but showed no further inhibitor effect on I-309 expression (Fig.5.4 A&B). Notably, the addition of SNOG alone had no effect on the chemokine expression levels in the time points analysed (Fig. 5.4 A&B). This data supports the hypothesis that the IFN-γ inhibition of chemokine expression is dependent on endogenously-derived NO while exogenous NO had variable effects, potentiating IFN-γ inhibition of MCP-1 but not I-309.

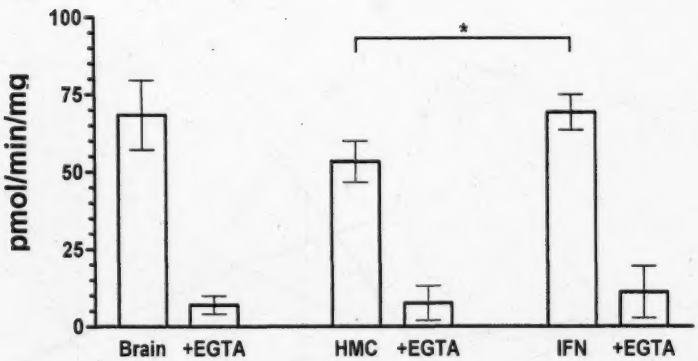


Fig. 5.3. Nitric oxide synthase (NOS) activity in HMC-1. NOS activity in untreated (0 h) and interferon-γ (IFN-γ)(18 h) stimulated HMC-1, as measured by the citrulline assay. The results are expressed as picomoles L-citrulline formed per minute, per mg of protein. Data shown as mean ± SEM for three independent experiments. *, Indicates $p < 0.05$.

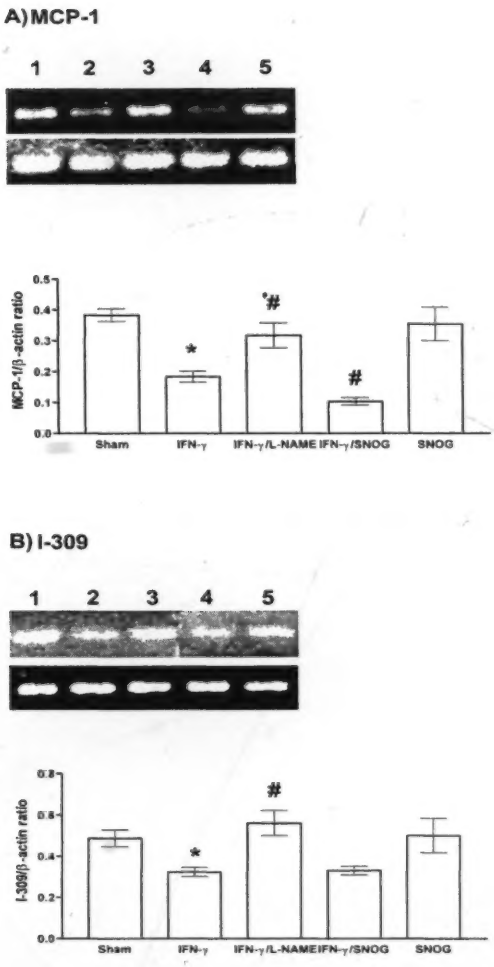


Fig. 5.4. Monocyte chemoattractant protein-1 (MCP-1)/I-309 mRNA regulation by interferon-γ (IFN-γ) and nitric oxide (NO). *A,* Measurement of messenger RNA (mRNA) levels for MCP-1 and β–actin by reverse-transcriptase polymerase chain reaction (RT-PCR) in HMC-1 cultured in media alone (1), with 200U/mL IFN-γ (2), with IFN-γ/L-NAME (100 μM) (3) with IFN-γ/SNOG (100 μM) and with SNOG (100 μM) alone (5)for 18 h. Densitometric analyses are shown as the MCP-1/β-actin ratio, expressed in arbitrary units. *B,* Measurement of mRNA levels for I-309 and β–actin by RT-PCR in HMC-1 cultured in media alone (1), with 200U/mL IFN-γ (2), with IFN-γ/L-NAME (100 μM) (3) with IFN-γ/SNOG (100 μM) and with SNOG (100 μM) alone (5) for 18 h. Densitometric analyses are shown as the I-309/β-actin ratio, expressed in arbitrary units. The PCR gels shown are representative of two others performed using HMC-1 from different cell isolations. Error bars represent SEM from three separate experiments. *, indicates $p<0.05$ compared to sham-treated cells; #, indicates $p<0.05$ compared to IFN-γ-treated cells.

Regulation of MCP-1 and I-309 release by NO

To determine if IFN-γ also effects MCP-1 and I-309 release from HMC-1, cells were treated with IFN-γ (200 U/mL, 18 h) then stimulated with PMA (30 ng/mL, 4 h). PMA has previously been shown to induce chemokine release from HMC-1 (18). HMC-1 constitutively release MCP-1 (693.3±150.7 pg/mL) and I-309 (343.3±67.6 pg/mL)(Fig. 5.5 A&B). Stimulation with PMA resulted in a significant ($p<0.05$) increase in MCP-1 (2090±205.9) and I-309 (2306.6±92.4) release compared to constitutive levels. Treatment with IFN-γ resulted in significant ($p<0.05$) inhibition of MCP-1 (47 %) and I-309 (38 %) respectively (Fig. 5.5 A&B).

To evaluate the role of NO, either L-NAME (NOS inhibitor) or SNOG (NO donor) were added concordantly with IFN-γ prior to PMA stimualtion. SNOG significantly ($p<0.05$) enhanced IFN-γ inhibition of chemokine release, while L-NAME removed the IFN-γ effect and in the case of MCP-1 marginally but not significantly enhanced release.

Discussion

IFN-γ is multifunctional cytokine with pleiotropic effects on various aspects of immune cell function (6). In rodents MC, IFN-γ has been shown to upregulate the expression of NOS with a concordant increase in NO formation which inhibits MC mediator secretion and adhesion (7,8). NO formed in this fashion acts as a downstream effector of IFN-γ. The role of IFN-γ in human MC is, however, much less defined. In this study we employed HMC-1 as a human MC model and utilized a cDNA microarray approach to identify genes differentially expressed by IFN-γ exposure. Among the genes down-regulated included the chemokines MCP-1 (CCL-2) and I-309 (CCL-1), and upregulated expression was detected for eNOS. Previously, in rat MC, we showed that IFN-γ upregulated iNOS mRNA and protein, but had no effect on eNOS expression (12). To our knowledge this is the first study to show IFN-γ alone upregulates mRNA for both constitutive cNOS (nNOS and eNOS) isoforms. Studies in astrocytes showed that treatment with IL-12, TNF and IFN-γ upregulated eNOS expression, though nNOS was not investigated (19).

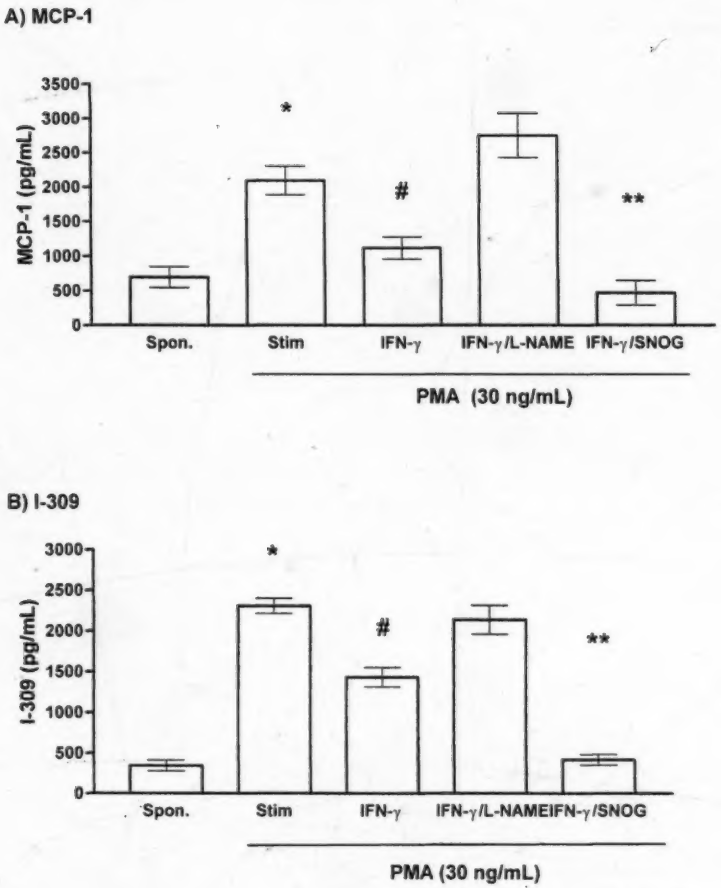


Fig. 5.5. Effect of nitric oxide synthase (NOS) inhibition and exogenous nitric oxide (NO) on monocyte chemoattractant protein-1 (MCP-1) and I-309 release in HMC-1. Concentrations of MCP-1 and I-309 in medium from HMC-1 was determined by ELISA. Cells were pre-incubated (18 h) with IFN-γ (200 U/mL) and NOS inhibitor (L-NAME, 100 μM) or NO donor (SNOG, 100 μM), then stimulated with phorbol myristate acetate (PMA) (30 ng/mL) for 4 h. Results are expressed as mean ± SEM for three independent experiments. *, Indicates $p < 0.05$ by comparison with sham-treated cells; #, indicated $p<0.05$ compared to PMA-stimulated cells; **, indicates $p<0.05$ compared to IFN-γ-treated cells.

Conversely, IFN-γ has been shown to down-regulate eNOS expression in endothelial cells (20). The eNOS promoter sequence is complex, with binding sites for numerous common transcription elements including NF-κB and AP-1 and studies show that eNOS can be modulated by numerous stimuli (21,22). However, the promoter region is also subject to multiple positive and negative transcription signals, and eNOS regulation is hypothesized to be complex and cell type specific as the above studies show (21). The fact that the nNOS mRNA levels in our study were increased but protein levels remained stable indicates that nNOS regulation may take place at both transcriptional and translational levels.

Other studies have confirmed some of our gene array results. Indeed, we showed the expected expression of c-kit and TNF as well as the urokinase receptor and NGF-R expression, results that have been shown by other reports (23,24). Furthermore, human MC also express high levels of both MCP-1 and I-309 mRNA upon activation through IgE (25). However, there are also some differences between previous expression analysis studies in human MC and our results. In human cord-blood derived MC, IL-8 expression has been shown to be IFN-γ regulated (26), though we saw no such effect in HMC-1. Methodological differences (gene array vs RNase protection assay) or the cell types involved may attribute to these discrepancies. Interestingly, the overall differential regulation of genes in HMC-1 by IFN-γ was small (~3%) which is similar to numbers seen in other studies in HMC-1 using larger arrays and other treatments (16).

Chemokines are a family of structurally related chemoattractant cytokines that play a central role during inflammation (27). In previous human MC studies, MCP-1 and I-309 expression and release were upregulated after activation (18,25). As MC are strategically located next to vessels and epithelial surfaces, such release after activation may account for the cellular influx of lymphocytes, neutrophils and eosinophils into asthmatic lung (4). Indeed, recent in-vivo studies in both humans and rodents show I-309 and MCP-1 as important mediators of the preferential attraction of Th2 cells in asthmatic inflammation (27,28). Given that endogenous NO has been shown to regulate chemokine expression in other cell types (29,30), and that NO formed in inflammatory conditions such as asthma may be a key

element in determining the cellular influx seen in this disease (31,32), our findings that IFN-γ-treated human MC upregulate eNOS resulting in decreased chemokine release suggests a critical role for IFN-γ in determining long-term environmental phenotype of MC in certain disease states.

In other cell types, NO regulates gene expression though modulating transcription factor activity (33,34). Previous studies in effector T-cells have shown that IFN-α inhibited I-309 expression and release, though the role of NO was not studied (35). As human eNOS has γ-activated sequences (GAS) in its promotor region and both Type I (α/β) and Type II (γ) interferon signaling pathways converge at STAT1 downstream of their respective receptors (17), NO-dependent IFN effects may be a common mechanism for the control of chemokine expression in immune cells. Indeed, the role for eNOS in regulating immune responses is evolving and recent studies show eNOS is necessary for propagating macrophage responsiveness (36), and defined a unique role for nNOS in a mouse model of asthma (37). As production of iNOS has not yet been noted in human MC populations, further study of nNOS/eNOS signaling pathways may be of clinical interest.

In this study we showed that inhibition of endogenous NO production in HMC-1 with L-NAME removed the IFN-γ induced mRNA down regulation of MCP-1 and I-309. Interestingly, addition of exogenous NO via a donor, SNOG, to IFN-γ treated cells potentiated the IFN-γ effect only on MCP-1 expression. Furthermore, addition of SNOG alone to HMC-1 had no effect on either MCP-1 or I-309 expression in the time frame studied. These results point to potential differences in the kinetics and localization of NO production in MC with resulting variable effects. This is further complicated by the lack of such differences noted in our chemokine release assays. Addition of L-NAME potentiated release of MCP-1 and I-309, while SNOG had an inhibitory effect. We have previously shown using live-cell confocal microscopy that HMC-1 endogenously produce NO in both cytoplasmic and nuclear sites (14). Thus endogenously derived NO may have effects on both gene expression and mediator release due to this pattern of subcellular targeting, while SNOG may only effect the mediator release aspect, due to half life of this donor, or lack of permeation to nuclear sites. Such differences in

effect have been noted in other studies of NO effects on chemokine expression (30,38), and stress the importance of the source of NO on the observed outcomes in cellular response.

It has previously been shown that NO can moderate MC mediator secretion (degranulation) (39), MC leukotriene production (14), and now we have presented evidence that MC-derived NO is upregulated by IFN-γ treatment and moderates chemokine expression and release. Thus NO appears to be a critical player in orchestrating MC responsive phenotype. Further study of MC NO mediated effects may yield new pharmacological tools to treat MC-associated diseases such as asthma.

References

1. Gurish, M.F. and Austen, K.F. 2001. The diverse roles of mast cells. *J. Exp. Med.* 194:F1-F5.

2. Wedemeyer, J., Tsai, M., and Galli, S.J. 2000. Roles of mast cells and basophils in innate and acquired immunity. *Curr. Opin. Immunol.* 12:624-631.

3. Benoist, C. and Mathis, D. 2002. Mast cells in autoimmune disease. *Nature* 420:875-878.

4. Brightling, C.E., Bradding, P., Pavord, I.D., and Wardlaw, A.J. 2003. New insights into the role of the mast cell in asthma. *Clin. Exp. Allergy* 33:550-556.

5. Schoeler, D., Grutzkau, A., Henz, B.M., Kuchler, J., and Kruger-Krasagakis, S. 2003. Interleukin-6 enhances whereas tumor necrosis factor alpha and interferons inhibit integrin expression and adhesion of human mast cells to extracellular matrix proteins. *J. Invest. Dermatol.* 120:795-801.

6. Shtrichman, R. and Samuel, C.E. 2001. The role of gamma interferon in antimicrobial immunity. *Curr. Opin. Microbiol.* 4:251-259.

7. Wills, F.L., Gilchrist, M., and Befus, A.D. 1999. Interferon-gamma regulates the interaction of RBL-2H3 cells with fibronectin through production of nitric oxide. *Immunology* 97:481-489.

8. Eastmond, N.C., Banks, E.M., and Coleman, J.W. 1997. Nitric oxide inhibits IgE-mediated degranulation of mast cells and is the principal intermediate in IFN-gamma induced suppression of exocytosis. *J. Immunol.* 159:1444-1450.

9. Okayama, Y., Kirshenbaum, A.S., and Metcalfe, D.D. 2000. Expression of a functional high-affinity IgG receptor, Fc gamma RI, on human mast cells: Up-regulation by IFN-gamma. *J. Immunol.* 164:4332-4339.

10. Salvi, S.S., Babu, K.S., and Holgate, S.T. 2001. Is asthma really due to a polarized T cell response toward a helper T cell type 2 phenotype? *Am. J. Respir. Crit. Care Med.* 164:1343-1346.

11. Alderton, W.K., Cooper, C.E., and Knowles, R.G. 2001. Nitric oxide synthases: structure, function and inhibition. *Biochem. J.* 357:593-615.

12. Gilchrist, M., Savoie, M., Nohara, O., Wills, F.L., Wallace, J.L., and Befus, A.D. 2002. Nitric oxide synthase and nitric oxide production in in vivo-derived mast cells. *J. Leukoc. Biol.* 71:618-624.

13. Kashiwagi, S., Kajimura, M., Yoshimura, Y., and Suematsu, M. 2002. Nonendothelial source of nitric oxide in arterioles but not in venules: alternative source revealed in vivo by diaminofluorescein microfluorography. *Circ. Res.* 91:e55-e64.

14. Clancy, R., Varenika, B., Huang, W., Ballou, L., Attur, M., Amin, A.R., and Abramson, S.B. 2000. Nitric oxide synthase/COX cross-talk: nitric oxide activates COX-1 but inhibits COX-2-derived prostaglandin production. *J. Immunol.* 165:1582-1587.

15. Gilchrist, M., MacDonald, A.J., Neverova, I., Ritchie, B., and Befus, A.D. 1997. Optimization of the isolation and effective use of mRNA from rat mast cells. *J. Immunol. Methods* 201:207-214.

16. Cho, S.H., Yao, Z., Wang, S.W., Alban, R.F., Barbers, R.G., French, S.W., and Oh, C.K. 2003. Regulation of activin A expression in mast cells and asthma: its effect on the proliferation of human airway smooth muscle cells. *J. Immunol.* 170:4045-4052.

17. Ramana, C.V., Gil, M.P., Schreiber, R.D., and Stark, G.R. 2002. Stat1-dependent and -independent pathways in IFN-gamma-dependent signaling. *Trends Immunol.* 23:96-101.

18. Selvan, R.S., Butterfield, J.H., and Krangel, M.S. 1994. Expression of multiple chemokine genes by a human mast cell leukemia. *J. Biol. Chem.* 269:13893-13898.

19. Barna, M., Komatsu, T., and Reiss, C.S. 1996. Activation of type III nitric oxide synthase in astrocytes following a neurotropic viral infection. *Virology* 223:331-343.

20. Wagner, A.H., Schwabe, O., and Hecker, M. 2002. Atorvastatin inhibition of cytokine-inducible nitric oxide synthase expression in native endothelial cells in situ. *Br. J. Pharmacol.* 136:143-149.

21. Fleming, I. and Busse, R. 2003. Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 284:R1-12.

22. Greenberg, S.S., Ouyang, J., Zhao, X., and Giles, T.D. 1998. Human and rat neutrophils constitutively express neural nitric oxide synthase mRNA. *Nitric. Oxide.* 2:203-212.

23. Cho, S.H., Tam, S.W., Demissie-Sanders, S., Filler, S.A., and Oh, C.K. 2000. Production of plasminogen activator inhibitor-1 by human mast cells and its possible role in asthma. *J. Immunol.* 165:3154-3161.

24. Tam, S.Y., Tsai, M., Yamaguchi, M., Yano, K., Butterfield, J.H., and Galli, S.J. 1997. Expression of functional TrkA receptor tyrosine kinase in the HMC-1 human mast cell line and in human mast cells. *Blood* 90:1807-1820.

25. Nakajima, T., Inagaki, N., Tanaka, H., Tanaka, A., Yoshikawa, M., Tamari, M., Hasegawa, K., Matsumoto, K., Tachimoto, H., Ebisawa, M., Tsujimoto, G., Matsuda, H., Nagai, H., and Saito, H. 2002. Marked increase in CC chemokine gene expression in both human and mouse mast cell transcriptomes following Fcepsilon receptor I cross-linking: an interspecies comparison. *Blood* 100:3861-3868.

26. Rizzo, M.A. and Piston, D.W. 2003. Regulation of beta cell glucokinase by S-nitrosylation and association with nitric oxide synthase. *J. Cell Biol.* 161:243-248.

27. Panina-Bordignon, P., Papi, A., Mariani, M., Di Lucia, P., Casoni, G., Bellettato, C., Buonsanti, C., Miotto, D., Mapp, C., Villa, A., Arrigoni, G., Fabbri, L.M., and Sinigaglia, F. 2001. The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. *J. Clin. Invest.* 107:1357-1364.

28. Bishop, B. and Lloyd, C.M. 2003. CC chemokine ligand 1 promotes recruitment of eosinophils but not Th2 cells during the development of allergic airways disease. *J. Immunol.* 170:4810-4817.

29. Walpen, S., Beck, K.F., Schaefer, L., Raslik, I., Eberhardt, W., Schaefer, R.M., and Pfeilschifter, J. 2001. Nitric oxide induces MIP-2 transcription in rat renal mesangial cells and in a rat model of glomerulonephritis. *FASEB J.* 15:571-573.

30. Muhl, H. and Dinarello, C.A. 1997. Macrophage inflammatory protein-1 alpha production in lipopolysaccharide-stimulated human adherent blood mononuclear cells is inhibited by the nitric oxide synthase inhibitor N(G)-monomethyl-L- arginine. *J. Immunol.* 159:5063-5069.

31. Thomassen, M.J., Raychaudhuri, B., Dweik, R.A., Farver, C., Buhrow, L., Malur, A., Connors, M.J., Drazba, J., Hammel, J., Erzurum, S.C., and Kavuru, M.S. 1999. Nitric oxide regulation of asthmatic airway inflammation with segmental allergen challenge. *J. Allergy Clin. Immunol.* 104:1174-1182.

32. Blease, K., Kunkel, S.L., and Hogaboam, C.M. 2000. Acute inhibition of nitric oxide exacerbates airway hyperresponsiveness, eosinophilia and C-C chemokine generation in a murine model of fungal asthma. *Inflamm. Res.* 49:297-304.

33. Natarajan, R., Gupta, S., Fisher, B.J., Ghosh, S., and Fowler, A.A. 2001. Nitric oxide suppresses IL-8 transcription by inhibiting c-Jun N- terminal kinase-induced AP-1 activation. *Exp. Cell. Res.* 266:203-212.

34. Kang, J.L., Park, W., Pack, I.S., Lee, H.S., Kim, M.J., Lim, C.M., and Koh, Y. 2002. Inhaled nitric oxide attenuates acute lung injury via inhibition of nuclear factor-kappa B and inflammation. *J. Appl. Physiol.* 92:795-801.

35. Iellem, A., Colantonio, L., Bhakta, S., Sozzani, S., Mantovani, A., Sinigaglia, F., and D'Ambrosio, D. 2000. Inhibition by IL-12 and IFN-alpha of I-309 and macrophage-derived chemokine production upon TCR triggering of human Th1 cells. *Eur. J. Immunol.* 30:1030-1039.

36. Connelly, L., Jacobs, A.T., Palacios-Callender, M., Moncada, S., and Hobbs, A.J. 2003. Macrophage endothelial nitric-oxide synthase autoregulates cellular activation and pro-inflammatory protein expression. *J. Biol. Chem.* 278:26480-26487.

37. De Sanctis, G.T., MacLean, J.A., Hamada, K., Mehta, S., Scott, J.A., Jiao, A., Yandava, C.N., Kobzik, L., Wolyniec, W.W., Fabian, A.J., Venugopal, C.S., Grasemann, H., Huang, P.L., and Drazen, J.M. 1999. Contribution of nitric oxide synthases 1, 2, and 3 to airway hyperresponsiveness and inflammation in a murine model of asthma. *J. Exp. Med.* 189:1621-1630.

38. Guo, H.T., Cai, C.Q., Schroeder, R.A., and Kuo, P.C. 2002. Nitric oxide is necessary for CC-class chemokine expression in endotoxin-stimulated ANA-1 murine macrophages. *Immunol. Lett.* 80:21-26.

39. Coleman, J.W. 2002. Nitric oxide: a regulator of mast cell activation and mast cell- mediated inflammation. *Clin. Exp. Immunol.* 129:4-10.

Chapter 6

IFN-γ Regulates Protein Inhibitor of Nitric Oxide Synthase in Human Mast Cells

Introduction

Mast cells (MC) are tissue-resident immune effector cells that arise from immature bone marrow precursors. They produce and secrete numerous bioactive agents and have been implicated in diverse homeostatic functions such as angiogenesis, wound healing and tissue remodeling (1). Due to their armamentarium of mediators and strategic localization, MC also play central roles in various disease states such as multiple sclerosis and T_H2-driven inflammatory conditions such as asthma (2).

The cytokine interferon-γ (IFN-γ) has been shown in numerous systems to down-regulate various T_H2-type MC responses (3). Numerous studies in rodents have shown that IFN-γ inhibits MC adhesion as well as modulating MC mediator secretion and cytokine production (4,5). The role that IFN-γ plays in regulating human MC is less defined, though recent studies have shown that IFN-γ may be important in sculpting MC phenotype by determining a distinct pattern of cytokine/chemokine expression (6).

In rodents, a major downstream effector of IFN-γ is the radical, nitric oxide (NO)(7). NO is derived from the amino acid L-arginine by the nitric oxide synthase (NOS) family of enzymes that are loosely classified based on Ca^{2+} dependency for activity. The Ca^{2+}-dependent members include endothelial (eNOS) and neuronal (nNOS), characterized by constitutive expression and low NO production. Inducible NOS (iNOS) is upregulated by a variety of inflammatory mediators and functions independently of cellular Ca^{2+} levels and releases large amounts of NO (8). Numerous investigators have shown that rodent MC are regulated by endogenous NO from both constitutive and inducible sources (5,9,10). We recently showed that human MC express the nNOS and eNOS isoforms and that these levels could be upregulated by IFN-γ (11). Interestingly, no iNOS was detected in human MC at the mRNA or protein level. Furthermore, we showed that human MC produce endogenously derived NO, and that this has a role in regulating leukotriene and chemokine release (12). However, little information is available concerning the regulatory pathways and protein-protein interactions that control human NOS localization and activity. Protein inhibitor of NOS (PIN), a

small (89 amino acid) protein, has recently been shown to bind all NOS isoforms and is widely expressed in human tissues (16). PIN has been shown to inhibit nNOS activity by dimer-interference, and is homologous to the 8 kDa dynein light chain (DLC). Dyneins are a large group of molecular motor proteins that are involved in retrograde transport along microtubules (17). Thus, the aim of the present study was therefore to investigate candidate genes such as PIN that may be involved in the regulation of human MC NOS by IFN-γ.

Materials and Methods

Reagents

The goat polyclonal antibody against PIN was obtained from Santa Cruz (Santa Cruz, CA) and mouse monoclonal against the same protein was from BD Transduction Laboratories (San Diego, CA).

Cell lines

HMC-1, an immature human mast cell line (a kind gift from J.H. Butterfield, Minneapolis, MN), was cultured in Iscove's medium (Life Technologies, Grand Island, NY), with 10% fetal bovine serum (FBS), 2 mM glutamine, 40 U/mL penicillin/streptomycin and 1.0 mM thioglycerol. Cells were harvested and fed every 3-4 days. Human intestinal mast cells (±IgE crosslinking) were provided by Dr. S. Bischoff.

Gene array

HMC-1 were treated with IFN-γ (200 U/mL) (R&D Systems, Minneapolis, MN) for 18 h. Total RNA was isolated using a modification of the Chomczynski/Sacchi method as previously described and compared against unstimualted HMC-1 by the use of cDNA expression array specific for human sequences (R&D Systems). RNA (5 μg) was used as the template for ^{32}P-dCTP-labeled probe synthesis. After hybridization for 18 h the array was washed and then exposed to X-ray film (Kodak) for 48 h at -80°C. The spot intensities were quantified by densitometry (SigmaGel, SPSS Science, Chicago, IL). Expression levels were measured as signal/background (negative control) ratio with a 2 fold-difference between populations considered as differentially regulated as previously described (13).

Reverse-transcriptase Polymerase Chain Reaction

Semi-quantitative RT-PCR was run as we have previously described (14). One μg of RNA was converted to cDNA by the reverse transcription reaction (M.MLv reverse transcriptase, GIBCO/BRL) in a total volume of 20 μL. PCR amplification was performed on a PTC-100 Thermal Cycler (MJ Research, Boston, MA). The primers were designed to be intron-spanning based on published sequence data: PIN, sense 5′-TTCTCCACGGTAACCATGTG-3′, antisense 5′-ACAACGTGGGCAGAAGTATG-3′ (PCR product, 520 bp); β-actin, sense 5′-GTGGGGCGCCCCAGGCACCA-3′, antisense 5′-GTCCTTAATGTCACGCACGATTTC- 3′ (PCR product, 526 bp). The number of cycles used for the primer sets was 25-35, (depending on the linear range). The conditions for PCR amplification were as follows: denaturing at 95°C for 45 s, annealing for 45 s, and extension at 72°C for 1 min. The optimized annealing temperature was 47°C for PIN and β-actin. Products were run on a 1.2 % agarose gel and stained with ethidium bromide (Sigma). Band intensities were obtained with a gel scanner (Imagemaster, Pharmacia, Denver, CO).

Western blot

MC were incubated in 24 well plates at 1 x 10^6 cells/well from 0 to 18 h in various experimental conditions. The cells were dissociated by 500 μL of RIPA buffer (PBS,1% NP-40). The total protein content of each sample was determined by the Bradford technique (BioRad). Fifteen μg of protein from each sample was mixed with Lamelli loading buffer containing SDS and β-mercaptoethanol. Samples were electrophoretically separated on a 12% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (BioRad). The membrane was then incubated with primary antibodies for 1 h at room temperature. Dilutions of the primary antibodies are; polyclonal PIN (0.5 μg/mL) and monoclonal PIN (1.0 μg/mL). The secondary antibody, HRP conjugated rabbit anti-goat or mouse IgG (Serotec, Raleigh, NC) was added to the membrane and incubated for 1 h at room temperature. Labelling was detected by chemiluminescence by addition of SuperSignal substrate solution (Pierce, Rockford, IL). The resulting bands were scanned and quantified in a gel scanner (ImageMaster DTS, Pharmacia).

Confocal Microscopy

Localization of PIN in MC was performed on HMC-1 fixed in 4% paraformaldehyde for 20 min, then permeablized with 0.1% triton X-100 in PBS. Slides were blocked for 30 min with 10% FBS and 3% BSA in PBS. All slides were incubated overnight at 4°C with primary antibody, mouse anti-PIN (2 μg/mL), rabbit anti-eNOS (0.5 μg/mL) and rabbit anti nNOS (2.0 μg/mL). Specific antibody binding was detected with rhodamine red-labeled rabbit anti-mouse or BODIPY- labeled goat anti-rabbit secondary antibodies (Molecular Probes). Negative controls with rabbit serum, were run concurrently. Cell images were obtained using a 40x 1.3 oil Plan-Neofluar objective on a Zeiss confocal laser scanning microscope (LSM510; Heidelberg, Germany).

Results

cDNA array analysis of IFN-γ regulated gene expression in HMC-1

IFN-γ is a well-known regulator of gene expression (3). To search for genes regulated by IFN-γ in human MC we performed cDNA expression array analysis in HMC-1. Of the 387 genes present on the array, HMC-1 RNA hybridized with 60 (60/387=15.5%) with IFN-γ upregulating seven (7/60=12%), downregulating 21 (21/60=35%) and 32 unchanged (32/60=53%). NO-related genes that were regulated by IFN-γ included the downregulation of PIN (-5.1 fold) and upregulation of eNOS (+4.7 fold)(Fig. 6.1).

Confirmation of IFN-γ regulation of mRNA expression

As there can be a moderate error rate with gene array analysis combined with lower sensitivity we looked to confirm the array results. Total RNA was extracted from unstimulated HMC-1, and from HMC-1 treated with IFN-γ (200 U/mL) for 18 h. eNOS and PIN mRNA production was assessed by RT-PCR vs β-actin (Fig. 6.2). As identified with the Array, levels of eNOS and PIN mRNA were up- or down-regulated respectively. No change in NOS mRNA was noted in unstimulated HMC-1 at similar time points, nor was any product detected in the absence of reverse transcriptase enzyme (data not shown). Results are from RNA obtained from three independent batches of HMC-1.

Differential regulation of PIN mRNA in IgE-stimulated human intestinal mast cells (HIMC)

Due to availability, we also screened MC derived from culture of HIMC precursors treated with and without crosslinking of IgE receptors. Total RNA was extracted from unstimulated HIMC, and from HIMC treated *in vitro* with human IgE (2 μg/ml, Serotec, Raleigh, NC) then stimulated with mouse anti-human IgE antibody (1 μg/mL, Serotec) for 4 h. PIN mRNA production was assessed by RT-PCR vs a β-actin housekeeping standard. HIMC treated for 4 h with anti-IgE significantly down-regulated PIN mRNA expression in all three donors studied (Fig. 6.2).

PIN protein

To confirm the RT-PCR results western blot analysis on IFN-γ treated and untreated HMC-1 extracts were investigated. Unstimulated HMC-1 constitutively produced large amounts of PIN (10 kDa)(Fig. 6.3). Interestingly, in some preparations PIN protein was detected as a doublet. Treatment with IFN-γ (200 U/mL) inhibited PIN protein expression in a time-dependent manner, with almost complete inhibition at 18 h. Untreated HMC-1 cultured during the same time showed no changes in PIN protein expression (data not shown).

PIN confocal

To determine the cellular localization pattern of PIN, laser scanning confocal microscopy was employed. Using this approach, HMC-1 stained strongly for PIN protein with a diffuse pattern present throughout the cytoplasm with some strong accumulations near the perinuclear region (Fig. 6.3). Few cells (< 5%) also showed strong nuclear accumulations of PIN in addition to the cytoplasmic staining. Cells stained in parallel with non-immune rabbit serum were also negative.

As PIN has been shown to bind to all NOS isoforms, colocalization in combination with nNOS and eNOS was employed to determine the extent of such interactions in HMC-1. We have previously shown that eNOS in HMC-1 has a predominant nuclear localization with weaker cytoplasmic staining, with regions of plasmamembrane accumulation (11). In addition, nNOS has also been shown in HMC-1 to have a weak cytoplasmic expression pattern. These results were

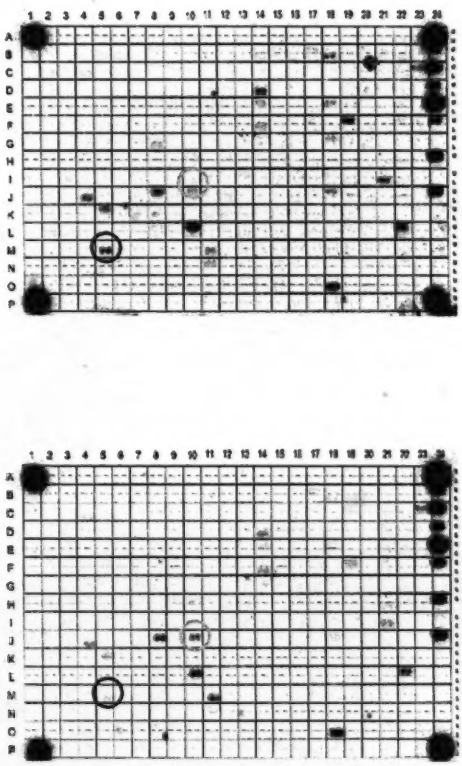


Fig. 6.1. cDNA array results. cDNA array analysis (R&D Systems, Human Cytokine) of hybridization experiment comparing cDNA generated from HMC-1 (top) or from interferon-γ (IFN-γ)(200 U/mL, 18 h) treated HMC-1. The array contains 387 genes, including 3 negative control cDNAs and 8 housekeeping genes. Genomic DNA is spotted in each corner to aid in grid alignment. Two genes differentially regulated between IFN-γ-treated and untreated HMC-1are marked by circles (red, PIN; blue, eNOS).

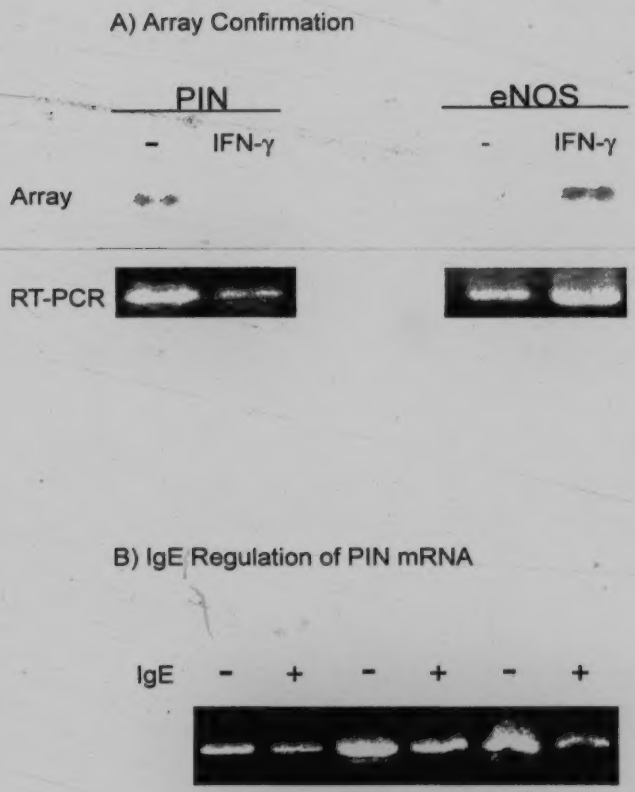


Fig. 6.2. Verification of differential expression of messenger RNA (mRNA) by gene-specific reverse-transcriptase polymerase chain reaction (RT-PCR). *A,* Results from gene array spots are shown directly above the RT-PCR products for both protein inhibitor of nitric oxide synthase (PIN) and endothelial nitric oxide synthase (eNOS) mRNA from HMC-1 treated with and without IFN-γ (200 U/mL, 18 h). *B,* PIN mRNA expression in human intestinal mast cells. Mast cells cultured *in-vitro* derived from intestinal tissue were treated with IgE, then crosslinked with anti-IgE for 4 h. RT-PCR analysis is from cells derived from three separate donors.

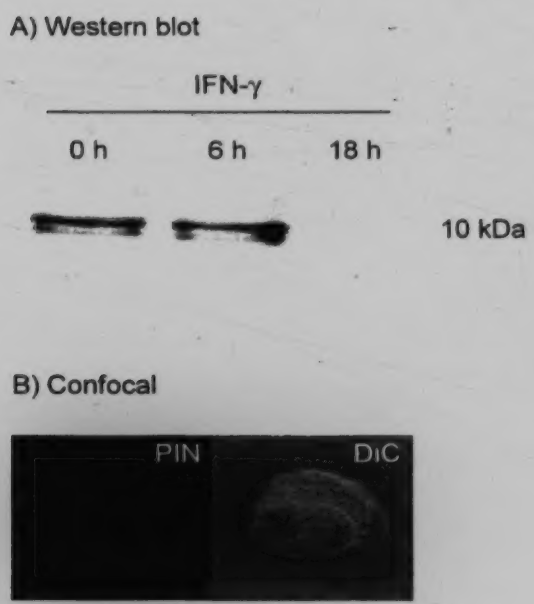


Fig.6.3. Protein inhibitor of nitric oxide synthase (PIN) protein expression in HMC-1. HMC-1 were lysed as described under "Materials and Methods"; proteins were separated by SDS-PAGE under reducing conditions. *A*, Representative immunoblot of PIN content in lysates obtained from HMC-1 treated with interferon-γ (IFN-γ)(200 U/mL) for the indicated times. Equal amounts of protein (10 μg) were loaded. Molecular weight markers indicated at right. All blots are representative of at least three independent experiments. *B*, HMC-1 were fixed in 4% paraformaldehyde and incubated with mouse anti-PIN antibody. Antibody labeling was detected with Rhodamine Red-conjugated rabbit anti-mouse antibodies (red). Original magnification x 800, bar= 10 μm.

confirmed in this study. When the PIN fluorescence signal (red) was overlayed with nNOS signal (green) there was small, yet discrete areas of colocalization, as indicated by the yellow fluorescence in the merged image, in the cytoplasm particularly near the cell surface and also in some cytoplasmic projections. Little colocalization was seen with eNOS protein. (Fig. 6.4).

Discussion

In this study we show for the first time the expression of PIN mRNA and protein in immune cells. Other studies have identified PIN protein expression, potentially in macrophages, however this was ancillary to the primary study on human muscle and was not clarified (15). PIN is highly conserved across eukaryotes, interacting with diverse proteins, and highly expressed as a cytoskeletal protein, therefore it is likely to be expressed in all cells (16).

Our results also indicate that PIN levels are regulated by the cytokine IFN-γ. Previous studies have shown that PIN levels can be upregulated by the products of COX-2 and may also vary depending on the stage of development, as PIN protein decreases in ventilator muscles beyond the embryonic stage (17,18). No link to IFN-γ regulation has been identified in other studies, and much of the promoter elements remain unknown.

However, IFN-γ has been shown to regulate other aspects of the cytoskelton in the control of cellular activation. Previously a role for endogenous IFN-γ has been shown in modulating epithelial polarity and tight junction formation (19). In addition, treatment with exogenous IFN-γ has been shown to modulate adhesion of monocytes through the regulation of filamentous actin and paxillin in the cytoskeleton (20). A role for NO and NOS in these process has been evolving in parallel, as we have previously shown that IFN-γ regulates MC adhesion in a NO-dependent fashion (4). In addition, more recent studies have defined an association of iNOS and eNOS with cortical actin, a pattern that is thought to be important in the localization and targeting of NO formation (21). Clearly IFN-γ is involved in the regulation and cross-talk between the cytoskeleton and NO, though any association between IFN-γ and regulation of NOS through binding or activity of

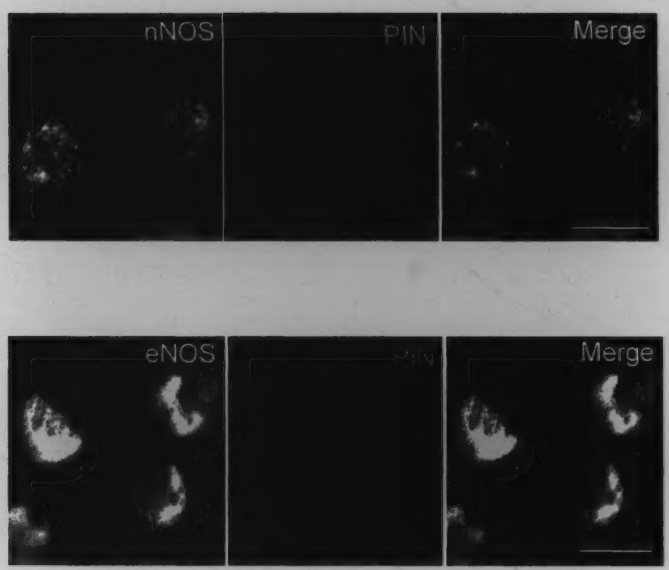


Fig.6.4. Colocalization of nitric oxide synthase (NOS) and protein inhibitor of nitric oxide synthase (PIN) in HMC-1. HMC-1 were fixed in 4% paraformaldehyde and incubated with mouse anti-PIN (red) and rabbit anti-endothelial NOS (eNOS) or neuronal NOS (nNOS)(green). Antibody labeling was detected with BODIPY-conjugated goat anti-rabbit and rhodamine-red-conjugated rabbit anti-mouse antibodies, overlapping fluorescence is indicated in yellow. Original magnification x 800, bar= 10 μm.

PIN remain to be determined.

PIN is homologous to the dynein light chain (DLC), which is a component of the dynein motor complex is responsible for protein and organelle transport along microtubules (22). Dyneins are large, multimeric complexes (1.2 mDa) consisting of two heavy chains, two or three intermediate chains, and one light chain (22). The role of these various light chains is unknown, though in *Drosophila*, loss-of-function mutants are associated with various morphological defects (23). Furthermore, since it was cloned, PIN has been identified as a binding partner for numerous and seemingly unrelated proteins, including myosin V, IκB, Dystrophin, p53, TRPS1 transcription factor, Bim, PTH, and NOS (24-28). This highlights the potential involvement of PIN in such diverse activities as locomotion, transcription and apoptosis. Interestingly, another critical component of NOS activity, calmodulin, also has similar diverse binding patterns (29). Our results showing that only a small fraction of cytoplasmic PIN binds to nNOS, and that PIN was also localizes to the nucleus in a small percentage of cells. This further collaborates this concept that other roles for PIN exist in MC beyond the binding and regulation of NOS.

The confocal data appear to support the hypothesis that PIN interacts predominantly with nNOS in MC. However, recent studies have shown that PIN has the potential to interact with all NOS isoforms (30). Further study employing immunoprecipitation or purified tagged proteins may be necessary to define the role of PIN binding to individual NOS species in human MC as well as to identify other potential PIN binding partners.

There has been emerging evidence that PIN plays a predominant role as a binding/adaptor molecule with NOS than as an inhibitor of NOS dimerization and resulting inactivation (31). As such, the finding of nNOS and PIN localization in MC by no means defines an inhibitory role. Previously we have shown that MC are heterogenous in their responses and NO production (11). Though all MC express NOS, the usage, activation and localization is clearly much more dynamic than has been initially assumed. Dyneins have been reported to be regulated by

redox state, and also can bind to inhibitors of PKA as well as Ca^{2+} molecules (32-34). As any number of these events could have a potential effect on NOS activity, the dynamic nature of NO formation and it's association with PIN expression and function needs further defining.

References

1. Metcalfe, D.D., Baram, D., and Mekori, Y.A. 1997. Mast cells. *Physiol. Rev.* 77:1033-1079.

2. Boyce, J.A. 2003. The role of mast cells in asthma. *Prostaglandins Leukot. Essent. Fatty Acids* 69:195-205.

3. Ramana, C.V., Gil, M.P., Schreiber, R.D., and Stark, G.R. 2002. Stat1-dependent and -independent pathways in IFN-gamma-dependent signaling. *Trends Immunol.* 23:96-101.

4. Wills, F.L., Gilchrist, M., and Befus, A.D. 1999. Interferon-gamma regulates the interaction of RBL-2H3 cells with fibronectin through production of nitric oxide. *Immunology* 97:481-489.

5. Eastmond, N.C., Banks, E.M., and Coleman, J.W. 1997. Nitric oxide inhibits IgE-mediated degranulation of mast cells and is the principal intermediate in IFN-gamma-induced suppression of exocytosis. *J. Immunol.* 159:1444-1450.

6. Tachimoto, H., Ebisawa, M., Hasegawa, T., Kashiwabara, T., Ra, C., Bochner, B.S., Miura, K., and Saito, H. 2000. Reciprocal regulation of cultured human mast cell cytokine production by IL-4 and IFN-gamma. *J. Allergy Clin. Immunol.* 106:141-149.

7. Mosser, D.M. 2003. The many faces of macrophage activation. *J. Leukoc. Biol.* 73:209-212.

8. Alderton, W.K., Cooper, C.E., and Knowles, R.G. 2001. Nitric oxide synthases: structure, function and inhibition. *Biochem. J.* 357:593-615.

9. Heywood, G.J. and Thomas, P.S. 2002. Nicorandil inhibits degranulation and TNF-alpha release from RBL-2H3 cells. *Inflamm. Res.* 51:176-181.

10. Koranteng, R.D., Dearman, R.J., Kimber, I., and Coleman, J.W. 2000. Phenotypic variation in mast cell responsiveness to the inhibitory action of nitric oxide. *Inflamm. Res.* 49:240-246.

11. Gilchrist, M., and Befus, A.D. 2003. Expression, localization and regulation of nitric oxide synthase (NOS) in human mast cells: effects on leukotriene production. *Submitted*

12. Gilchrist, M., and Befus, A.D. 2003. IFN-γ regulates chemokine expression and release in human mast cells: role of nitric oxide. *Submitted.*

13. Cho, S.H., Tam, S.W., Demissie-Sanders, S., Filler, S.A., and Oh, C.K. 2000. Production of plasminogen activator inhibitor-1 by human mast cells and its possible role in asthma. *J. Immunol.* 165:3154-3161.

14. Gilchrist, M., Savoie, M., Nohara, O., Wills, F.L., Wallace, J.L., and Befus, A.D. 2002. Nitric oxide synthase and nitric oxide production in in vivo-derived mast cells. *J. Leukoc. Biol.* 71:618-624.

15. Guo, Y., Petrof, B.J., and Hussain, S.N. 2001. Expression and localization of protein inhibitor of neuronal nitric oxide synthase in Duchenne muscular dystrophy. *Muscle Nerve* 24:1468-1475.

16. Kamal, A. and Goldstein, L.S. 2002. Principles of cargo attachment to cytoplasmic motor proteins. *Curr. Opin. Cell Biol.* 14:63-68.

17. Chang, Y.W., Jakobi, R., McGinty, A., Foschi, M., Dunn, M.J., and Sorokin, A. 2000. Cyclooxygenase 2 promotes cell survival by stimulation of dynein light chain expression and inhibition of neuronal nitric oxide synthase activity. *Mol. Cell Biol.* 20:8571-8579.

18. Guo, Y., Greenwood, M.T., Petrof, B.J., and Hussain, S.N. 1999. Expression and regulation of protein inhibitor of neuronal nitric oxide synthase in ventilatory muscles. *Am. J. Respir. Cell Mol. Biol.* 20:319-326.

19. Marcelo, P. and Lefevre, F. 2002. Induction of the IFN-gamma gene and protein is linked to the establishment of cell polarity in a porcine epithelial cell line. *Exp. Cell. Res.* 280:33-44.

20. Surin, B., Rouillard, D., and Bauvois, B. 2000. Loss of alpha5beta1-mediated adhesion of monocytic cells to fibronectin by interferons beta and gamma is associated with changes in actin and paxillin cytoskeleton. *Hematol. J.* 1:172-180.

21. Webb, J.L., Harvey, M.W., Holden, D.W., and Evans, T.J. 2001. Macrophage nitric oxide synthase associates with cortical actin but is not recruited to phagosomes. *Infect. Immun.* 69:6391-6400.

22. King, S.M. 2000. The dynein microtubule motor. *Biochim. Biophys. Acta.* 1496:60-75.

23. Herzig, R.P., Andersson, U., and Scarpulla, R.C. 2000. Dynein light chain interacts with NRF-1 and EWG, structurally and functionally related transcription factors from humans and drosophila. *J. Cell Sci.* 23:4263-4273.

24. Jaffrey, S.R. and Snyder, S.H. 1996. PIN: an associated protein inhibitor of neuronal nitric oxide synthase. *Science* 274:774-777.

25. Magee, T.R., Ferrini, M.G., Davila, H.H., Zeller, C.B., Vernet, D., Sun, J., Lalani, R., Burnett, A.L., Rajfer, J., and Gonzalez-Cadavid, N.F. 2003. Protein inhibitor of nitric oxide synthase (NOS) and the n-,ethyl-d-aspartate receptor are expressed in the rat and mouse penile nerves and colocalize with penile neuronal NOS. *Biol. Reprod.* 68:478-488.

26. Kaiser, F.J., Tavassoli, K., Van den Bemd, G.J., Chang, G.T., Horsthemke, B., Moroy, T., and Ludecke, H.J. 2003. Nuclear interaction of the dynein light chain LC8a with the TRPS1 transcription factor suppresses the transcriptional repression activity of TRPS1. *Hum. Mol. Genet.* 12:1349-1358.

27. Giannakakou, P., Sackett, D.L., Ward, Y., Webster, K.R., Blagosklonny, M.V., and Fojo, T. 2000. p53 is associated with cellular microtubules and is transported to the nucleus by dynein. *Nat. Cell Biol.* 2:709-717.

28. Tang, Q., Staub, C.M., Gao, G., Jin, Q., Wang, Z., Ding, W., Aurigemma, R.E., and Mulder, K.M. 2002. A Novel Transforming Growth Factor-beta Receptor-interacting Protein That Is Also a Light Chain of the Motor Protein Dynein. *Mol. Biol. Cell.*13:4484-4496.

29. Haeseleer, F. and Palczewski, K. 2002. Calmodulin and Ca^{2+}-binding proteins (CaBPs): variations on a theme. *Adv. Exp. Med. Biol.* 514:303-17.

30. Hemmens, B., Woschitz, S., Pitters, E., Klosch, B., Volker, C., Schmidt, K., and Mayer, B. 1998. The protein inhibitor of neuronal nitric oxide synthase (PIN): characterization of its action on pure nitric oxide synthases. *FEBS Lett.* 430:397-400.

31. Rodriguez-Crespo, I., Straub, W., Gavilanes, F., and Ortiz de Montellano, P.R. 1998. Binding of dynein light chain (PIN) to neuronal nitric oxide synthase in the absence of inhibition. *Arch. Biochem. Biophys.* 359:297-304.

32. Shea, T.B. and Flanagan, L.A. 2001. Kinesin, dynein and neurofilament transport. *Trends Neurosci.* 24:644-648.

33. Yu, J., Yu, L., Chen, Z., Zheng, L., Chen, X., Wang, X., Ren, D., and Zhao, S. 2002. Protein inhibitor of neuronal nitric oxide synthase interacts with protein kinase A inhibitors. *Brain Res.* 99:145-149.

34. Rodriguez-Crespo, I., Yelamos, B., Roncal, F., Albar, J.P., Ortiz de Montellano, P.R., and Gavilanes, F. 2001. Identification of novel cellular proteins that bind to the LC8 dynein light chain using a pepscan technique. *FEBS Lett.* 503:135-141.

Chapter 7

General Discussion and Conclusions

I. Summary of data

The reactive radical, NO, controls several important aspects of MC biology, including adhesion and degranulation. The overall objectives of this thesis were to define NOS expression, regulation and the specific roles of NO in diverse MC populations. NOS mRNA and protein for all three isoforms were found in either rat (iNOS and eNOS) or human (nNOS and eNOS) MC respectively. Furthermore, MC can produce NO. iNOS expression was detected in rat MC only, by stimulation with anti-CD8 antibody, IgE-crosslinking or IFN-γ. While no iNOS could be found in human MC types studied, nNOS and eNOS mRNA and eNOS protein could be upregulated by IFN-γ. Gene array studies investigating IFN-γ regulation of human MC identified several novel and potentially NO-related targets including the chemokines MCP-1 and I-309 as well as the protein inhibitor of NOS (PIN). NO is known to inhibit MC granule mediator secretion, and we directly confirmed using live-cell confocal microscopy that endogenous MC NO formation is associated with inhibition of degranulation. MC NO formation was regulated by availability of the cofactor BH_4 and inhibition of this pathway lead to a hyperresponsive phenotype. MC-derived NO also regulated leukotriene production and chemokine expression and release. These varied effects can be associated with the cellular localization of NOS and NO in these cells.

II. NOS expression and heterogeneity in MC

MC are known to be modulated by NO and MC can also be a source of NO and express of NOS (1). Table 7.I provides a summary of the expression of NOS isoforms in MC. MC are capable of expressing all NOS isoforms, and the expression varies among the MC types and treatments used.

MC incubated with IFN-γ-activated peritoneal cells, which include NO-producing macrophages, are hyporesponsive and release little histamine in response to IgE-activation (2). This highlights the potential contribution of contaminating macrophage NO in in-vivo purified MC responses. Identification of the presence of NOS in MC has

Table 7.I Nitric oxide synthase expression in mast cells

Mast Cell		NOS	Phenotype	Conditions	Ref
Rat	PMC [a]	eNOS	CTMC	Constitutive	(6)
		nNOS	CTMC	Constitutive	(7)
		iNOS	CTMC	(I) IFN-γ, CD8, Ag	(6)
	Cerebral	eNOS	CTMC	Constitutive	(3)
	Muscle	iNOS	CTMC	Ischemia/reperfusion	(4)
	BMMC [b]	eNOS	MMC	Constitutive (IL-3)	np
	IMMC [c]	eNOS	MMC	Constitutive	np
	RBL [d]	eNOS	MMC ?	Constitutive, (I) IFN-γ	np
	RCMC [e]	eNOS	CTMC ?	Constitutive, (I) IFN-γ	np

Mast Cell		NOS	Phenotype	Conditions	Ref
Mouse	BMMC	eNOS	MMC	Constitutive (IL-3)	np
	BMMC	iNOS	CTMC	Constitutive (IL-3/SCF)	(8)
	BMMC	iNOS	CTMC	Constitutive (IL-3/SCF)	np
	Uterus	iNOS	CTMC ?	Pregnancy	(84)
	Muscle	iNOS	CTMC ?	Ischemia/reperfusion	(36)
	P815 [f]	iNOS	?	TNF	(85)

Mast Cell		NOS	Phenotype	Conditions	Ref
Human	Skin	nNOS/eNOS	MC^{TC}	Constitutive	np
		nNOS	MC^{TC}	Constitutive	(5)
	Mucosal	nNOS	MC^{TC}	Constitutive	(9)
	Peribiliary	iNOS		Liver cirrhosis	(19)
	IMMC	nNOS/eNOS *	MC^{T}/MC^{TC} ?	Constitutive (IL-3, SCF)	np
	CBMC	eNOS	MC^{T}	Constitutive	np
	KU812 [g]	eNOS	basophilic	Constitutive, (I) IFN-γ	np
	HMC-1 [h]	nNOS/eNOS	MC^{TC} ?	Constitutive, (I) IFN-γ	np
	LAD2 [i]	eNOS	MC^{TC} ?	Constitutive	np

a, peritoneal mast cell; b, bone marrow mast cell; c, intestinal mucosal mast cell; d, rat basophilic leukemia; e, rat cultured mast cell; f, mouse mast cell line; g, human basophilic cell line; h, human mast cell line; i, mature human mast cell line. (I), inducible; np, not published; * 3/10 donors $nNOS^{+}$, 5/10 $eNOS^{+}$.

been fundamental in delineating this problem. NOS has been identified in rat cerebral MC (eNOS) (3), skeletal muscle MC (iNOS) (4) and human skin (nNOS) (5) by immunohistochemistry. We employed *in-situ* RT-PCR to directly identify iNOS mRNA in activated rat PMC (6). In addition, immunofluorescence localization has helped determine not only the presence of NOS in MC, but also the spatial distribution. There is now abundant evidence that MC produce NOS and can be a source of NO.

While few consistant patterns of NOS expression within MC subtypes are apparent, there are some notable characteristics. All MC express a cNOS isoform, though there is some apparent disagreement in the literature about expression of eNOS/nNOS. Indeed, data in Chapter II shows that rat PMC constitutively express eNOS mRNA, and confocal and western blot analysis showed eNOS expression in rat PMC, RBL-2H3 and RCMC cell lines (M. Gilchrist, unpublished). However, recent work by Kashiwagi et al showed predominant nNOS expression by flow cytometry in PMC derived from the same strain of rat (7). Despite this discrepancy, a constitutive source of NO in MC is the predominant theme. Thus, constitutive NOS activity in all MC is likely to be tightly controlled, as both nNOS and eNOS are modulated by numerous translational and post-translational controls, including the requirement for Ca^{2+}.

NOS expression does not appear to directly correlate with well-recognized MC subsets, CTMC and MMC or MC^{TC} and MC^{T}. That is, there is not exclusive expression of a particular isoform in a particular phenotype. Despite this, some interesting patterns exist with regard to the apparent presence of two constitutively expressed NOS isoforms in some MC populations. Bidri et al identified iNOS expression in mouse BMMC cultured in the presence of SCF and IL-3, these cells are known to express characteristics similar to CTMC. However, BMMC cultured in IL-3 alone were iNOS negative, with these cells expressing some characteristics of immature MMC (the alcian-blue-safranin technique showed blue staining granules presumed to reflect a lack of heparin)(8). Under similar culture conditions we saw that mouse BMMC grown in SCF/IL-3 (CTMC) were $eNOS^{+}/iNOS^{+}$ (n=2)

double positive by RT-PCR. Unfortunately we have not looked at eNOS expression in mouse BMMC cultured in IL-3 alone, though *in-vivo* derived rat IMMC (~75% pure) were $eNOS^{+}/iNOS^{-}$ (n=2) (M. Gilchrist, unpublished). Therefore, in some MC subpopulations conditions can be present that allow constitutive co-expression of two NOS isoforms and may be associated with MMC and CTMC heterogeneity. Though whether this co-expression has a direct role in determining or maintaining phenotype, or plays an accessory role resulting from differing culture or microenvironmental influences requires more study. Interestingly, cultures derived from BMMC of iNOS -/- mice are difficult to grow and propagate (Dr. C. Hesslinger, personal communication).

Such a parallel may also exist in humans, as HMC-1 as well as MC obtained from skin and some samples from *ex-vivo* derived intestinal MC co-express nNOS in addition to eNOS (5,9).

SCF is not only essential for MC development but also for normal dermal homeostasis and the production of interstitial cells of Cajal (ICC) in the gut (10). While there is no direct evidence of SCF transcriptionally regulating nNOS expression, ICC in rat and human intestine, as well as pigmented lesions in the skin of humans have all been shown to be nNOS positive (11,12). Furthermore, malignant dermal lesions such as melanoma or eosinophils found in dermal inflammatory sites are also nNOS positive (11,13). Thus, SCF may also elicit nNOS expression in certain settings though the distinct role for nNOS is unknown. Additionally, HMC-1 have a constitutively active mutation in the c-kit receptor and this may account for nNOS expression in these cells (14).

Though exceptions to this hypothesis are apparent, it is interesting to postulate that prolonged exposure to SCF, or combinations of SCF and other site-specific factors (i.e. cytokines, chemokines) changes expression patterns of NOS isoforms in MC. These patterns may contribute to the phenotypic variability in MC. Further studies to describe this phenomenon and define the role for the expression of an extra NOS besides eNOS already present are required (Fig. 7.1).

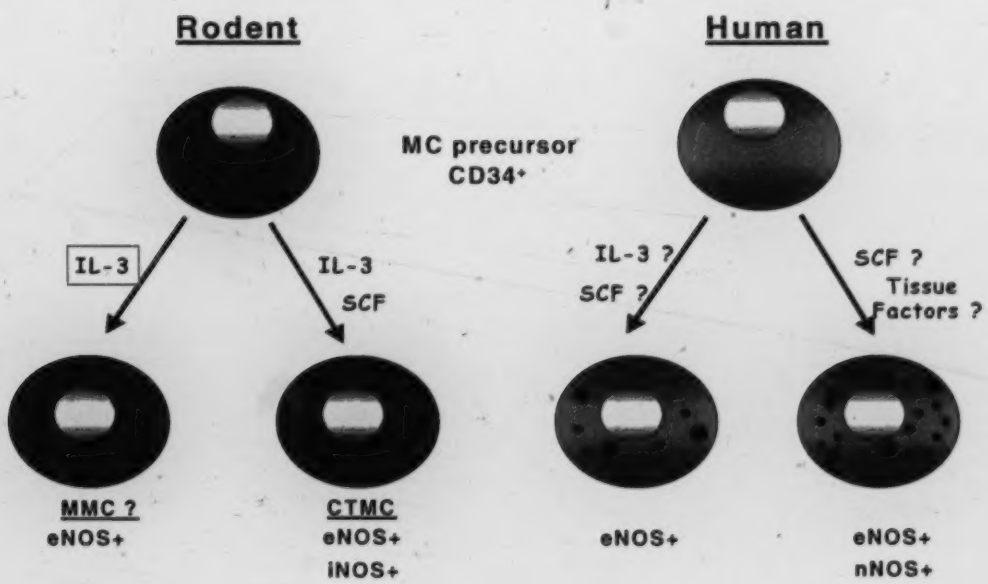


Fig. 7.1. Model of nitric oxide synthase (NOS) expression in mast cell heterogeneity. In rodent mast cell culture systems, incubation in the presence of IL-3 alone gives rise to a endothelial NOS $(eNOS)^+$/inducible NOS $(iNOS)^-$ population, while culture with both IL-3 and stem cell factor (SCF) gives rise to a $eNOS^+/iNOS^+$ population. A similar system may be present in human mast cells, as some populations are neuronal NO $(NOS)^+/eNOS^+$ while others express only eNOS. Such expression may be influenced by cytokines and SCF in particular, possibly in combination with other culture/microenvironmental factors.

One of the objectives of this thesis was to investigate iNOS expression in rat PMC. IFN-γ and anti-CD8 antibody induced iNOS expression in PMC, and this was approximately 2X greater than in MC stimulated by crosslinking of IgE (Chapter II). Similar treatments in rat MC lines (RCMC and RBL-2H3) did not induce iNOS expression, although eNOS could be increased by IFN-γ.

There is, however, poor correlation between expression of iNOS in rodent and human immune cells. Human monocytes and macrophages are poor producers of iNOS and NO *in-vitro* (15). Results in Chapter IV and V extended these observations, as no human MC population we studied expressed iNOS. The human iNOS promoter is significantly more complex than that in rodents, and this may contribute to the difficulty in identifying iNOS expression in humans (16,17). Indeed, identification of iNOS in human macrophages is most easily identified following isolation of these cells from inflammatory tissue (18). A similar scenario may exist with human MC, as one study has identified iNOS expression in liver MC during cirrhosis (19). As such, replication of the precise cellular environment needed for iNOS expression *in-vitro* remains unresolved and further investigation is required to conclusively define iNOS expression in *in-vivo* derived human MC.

The data in this thesis contribute to an emerging understanding of the role of nNOS/eNOS in immune cell regulation. Historically, tonic levels of NO derived from cNOS were thought to possess only "physiological" relevance, while a high level of NO derived from iNOS was a major mediator of "pathology" (20). However, this paradigm is beginning to change as new tools become available. For example, recent studies using eNOS -/- mice show blunted macrophage activation after LPS stimulation, likely though decreased cGMP production (21). Studies in a model of mouse asthma showed that nNOS contributes to airway hyperresponsiveness and cellular influx, an association that was not shown for iNOS -/- mice (22). Thus a better understanding of the roles of NOS in inflammation is developing.

III. <u>Defining NO production in mast cells</u>

NO has long been known as a moderator of MC responsiveness. What has been contentious is the source of NO and whether MC synthesize NO. Part of the

debate arises from the earlier lack of direct detection of NO from MC. A wide variety of methodologies have been employed to identify NO formation in MC, including downstream readouts of NO formation, such as cGMP levels (23) and inhibition of platelet aggregation (24). These approaches showed that rat MC produce an NO-like factor that inhibits histamine and PAF release (23,24). However, NO formation in these studies was by association, as they involved a complex signaling cascades that may result in formation of mediators other than NO that can cause the observed effects. Non-withstanding, these experiments revealed the possible presence of an active cNOS in some MC.

Several groups, including our own, have used the Griess assay, to detect the stable breakdown product of NO, nitrate (NO_2^-)(6,25). However, this assay does not specifically detect NO and buffer constituents or cell redox state can affect the results. Moreover, the assay lacks sensitivity, which may have contributed to conflicting results in the literature. Thus, data employing this assay must be approached cautiously, and corroboration with other methods is needed.

Detection of the conversion of radioactive arginine to citrulline is the basis for the Citrulline assay and offers a sensitive (pM) and specific method to quantify NOS activity (26). Results with PMC (Chapter II) and HMC-1 (Chapter IV and V) show for the first-time that cNOS activity is constitutively present, and that significant iNOS activity appears after IFN-γ treatment of PMC (Chapter II). There are potential drawbacks however, since the assay measures total NOS activity and as all cofactors are present in the reaction mix, no information on the dynamics of NO formation is deduced. Furthermore, the assay cannot dissect nNOS from eNOS activity. Since cNOS can be modulated on multiple levels, these results give only partial insight into NO formation in heterogeneous MC populations (27).

The Griess and Citrulline assays measure responses that represent an "average" across an entire population and do not identify the individual cells involved. Recently, fluorescent molecules specific for NO have been developed, including diaminofluorescene (DAF)(28). DAF positivity in live-cell confocal analysis has been associated with insulin release in individual pancreatic β-cells (29) and eNOS activity in endothelial cells and intact lung (30,31). This probe is a

powerful tool to determine the amount, timing and location of NO production in cells (see below).

IV. Compartmentalization and roles of NOS and NO in mast cells

NO is freely diffusible in biological systems and is highly reactive, characteristics that make defining specific protein targets and overall roles in cell regulation difficult. One mechanism that helps provide specificity to the actions of NO is its compartmentalization in the cell (32). For example, endothelial cells release NO from eNOS that is localized in the plasma membrane, a topography determined and regulated by caveolin binding, which controls when and where NO is released (33). Furthermore, bacterial phagocytosis in macrophages results in a surprising localization of iNOS in cortical regions, not in phagosomes (34), which is postulated to allow NO release to occur adjacent to the cell thereby avoiding toxic effects of intracellular NO formation. However, no studies investigating the topographical localization of NOS/NO in MC exist.

Sustained NO: Localization and targets: Most studies that have characterized the presence of NOS in MC, do not describe the intracellular localization (5,35,36). In Chapter II, immunohistochemistry localized iNOS protein to the cytoplasm of rat PMC after IFN-γ treatment (18 h). Some cells also showed granule association of the protein, as previously noted in rat skeletal muscle MC (4). This cytoplasmic iNOS localization has been previously noted in macrophages and implies a role in regulating cytoplasmic functions, or for extracellular release (34). Furthermore, live-confocal analysis in DAF-loaded, IFN-γ-treated PMC showed a predominant cytoplasmic localization of NO with some Golgi or perinuclear accumulation, confirming that iNOS can have a cytoplasmic localization in rat PMC (Chapter III).

Long-term treatment of HMC-1 with IFN-γ (18 h) upregulated nNOS and eNOS mRNA and eNOS protein. Such regulation of cNOS expression has been previously shown in rat MC lines (RBL-2H3 and RCMC)(37). This increase in NOS expression appears to also correlate with roles for NO in regulating chemokine transcription and release in HMC-1 and regulation of adhesion in RBL-2H3.

We have recently shown that NO is capable of nitrosylating and inhibiting calpain, a critical protease in adhesion complex formation (38). In addition the GTPase, Rac, is nitrosylated in neutrophils and has a modulatory role in granule release (39), a mechanism that may also effect exocytosis of MC granules. As IFN-γ treatment in rodent MC inhibits MC adhesion (RBL-2H3)(37), as well as degranulation (PMC)(40), eNOS (RBL-2H3) or iNOS (PMC) appear optimally located to contribute to these cytoplasmic-based effects (Fig. 7.2). Moreover, these effects appear to be independent of cGMP formation, indicating that no obvious NO-mediated activation of sGC takes place. Thus, effects of NO, though nitrosylation of other cellular proteins, may be the predominant mechanism.

No studies were done on IFN-γ effects on HMC-1 granule mediator release. However, IFN-γ inhibited both expression and release of the chemokines MCP-1 and I-309 from HMC-1 (Chapter V). DAF localization was not investigated in these IFN-γ-treated cells. However, untreated HMC-1 have cytoplasmic and nuclear DAF positivity that correlates with nNOS/eNOS localization. Thus, NO formation in the cytoplasm may modulate chemokine release, while NO in the nucleus can have well-known effects on regulation of gene-transcription. (Fig. 7.2)

Immediate NO: Localization and targets: Much like iNOS in rodents, nNOS protein localizes predominantly to cytoplasmic fractions in human MC. Due to variable and often low levels of expression, roles for nNOS in MC are difficult to define, especially because there is overlap with eNOS localization in these cells. Thus, antisense or other approaches may be necessary to delineate the role of nNOS in these cells. In neuronal systems, nNOS is found in large protein complexes involving nerve signaling and ion channel regulation (41). Extrapolation to MC function is difficult and more detailed studies are necessary.

Confocal analysis of endothelial cells expressing both wild type and acylation mutants of eNOS defined plasma membrane compartmentalization of eNOS (42). Other groups have also identified perinuclear, cytoplasmic and Golgi eNOS compartments in endothelial cells (43-45). eNOS expression in both rat and

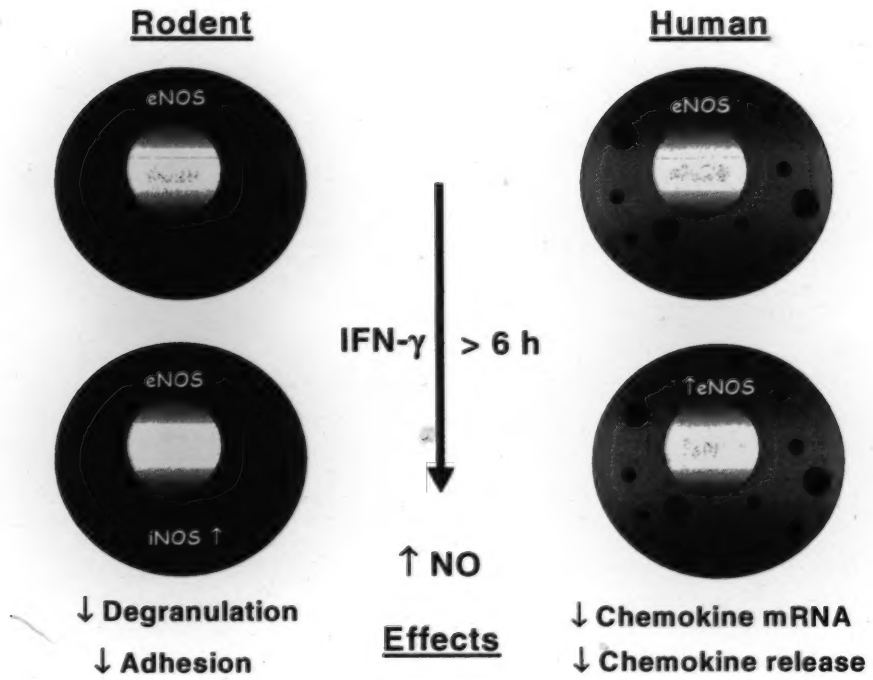


Fig. 7.2. Long-term (hours) effects of upregulated nitric oxide synthase (NOS) activity on mast cell responses. In rodent mast cells interferon-γ (IFN-γ) treatment upregulates endothelial NOS (eNOS) (RBL-2H3) or inducible NOS (iNOS) (peritoneal mst cell, PMC) resulting in predominant formation of nitric oxide in the cytoplasm. Nitric oxide (NO) effects cytoplasmic targets resulting in inhibition of adhesion and degranulation. In human mast cells, IFN-γ results in increased eNOS expression in both cytoplasmic and nuclear sites. The resulting NO formation in these regions targets nuclear factors that control chemokine mRNA expression, and cytoplasmic targets that control chemokine release.

human MC was surprisingly localized in a predominant nuclear pattern, with weak cytoplasmic and plasma membrane staining (Chapter IV and unpublished). Furthermore, these sites were defined as actively producing NO by DAF staining. Subcellular fractionation combined with western blot analysis confirmed these results. In addition, activation of DAF-loaded rat PMC by cross-linking IgE resulted in a dynamic change in the pattern of NO production. Approximately 30% of PMC showed Ca^{2+}-dependent NO formation within 5 min that localized to the cytoplasm, while some cells also become strongly positive in nuclear regions (approximately 60% of NO^+ PMC).

A role of nuclear eNOS activity was identified by parallel comparison to 5-lipoxygenase (5-LO), a protein known to be associated with the nuclear membrane in MC (46). Interestingly, eNOS shifted from the cytoplasm to the nucleus upon stimulation, a process previously defined for 5-LO. Thus, eNOS was in juxtaposition with 5-LO, a known target for NO regulation. Furthermore, inhibition of NO enhanced, while NO donors inhibited leukotriene production and release in a cGMP-independent manner. Studies in LPS-treated macrophages have shown NO-mediated nitrosylation and inhibition of 5-LO (47), and outlined a possible target for nuclear eNOS/NO in MC (Fig. 7.3).

A role for cytoplasmic eNOS is difficult to define due to the predominant nuclear shift noted above, though some plasma membrane expression of eNOS was also apparent. As previously noted, eNOS colocalizes with caveolin in endothelial cells. Mouse MC have been shown to express caveolin (48). This is a novel finding of this protein in immune cells, that otherwise seem bereft of these cellular structures. However, due to dual acylation, eNOS can also localize to lipid rafts, areas of high lipid order and cholesterol content that are rich in signaling proteins (49,50). In cell types where caveolin is not present, lipid raft interactions may represent a major element of plasma membrane eNOS localization and activation. Studies of the structure of lipid rafts and their molecular content will be of benefit in MC to define a potential regulatory mechanism for eNOS in these cells. Therefore, cytoplasmic eNOS may have similar roles to that of iNOS in PMC, in the regulation of adhesive or secretory mechanisms (Fig. 7.3).

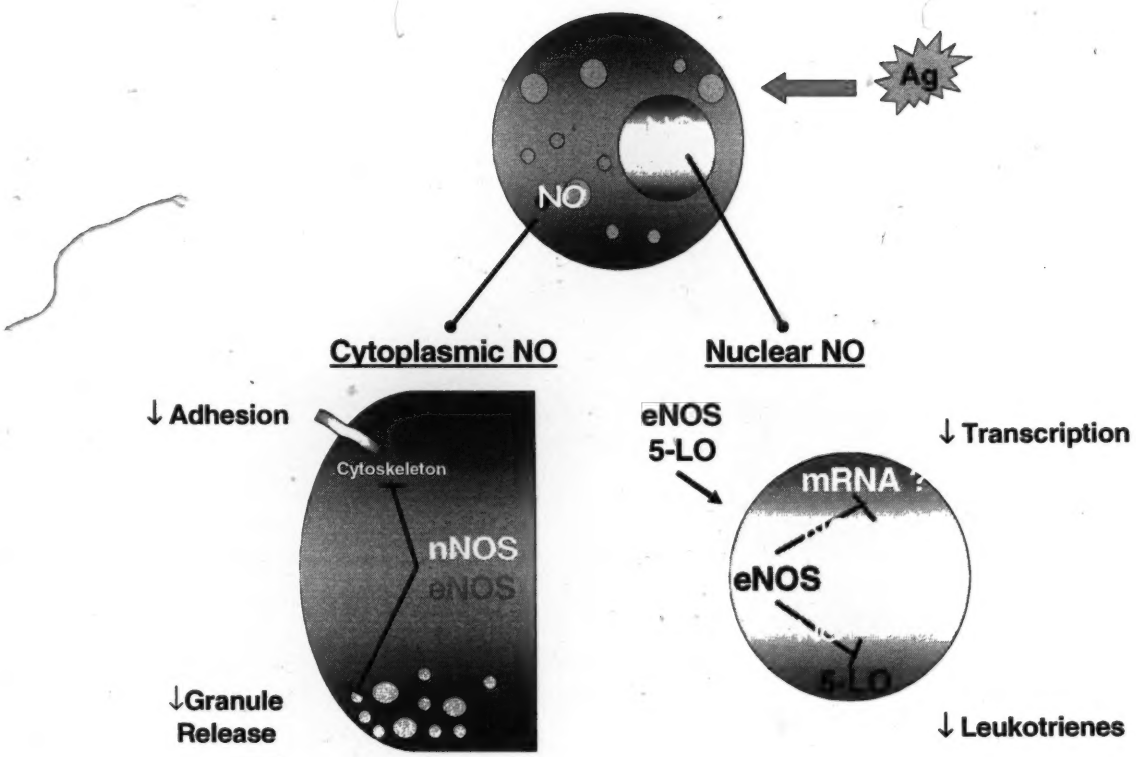


Fig. 7.3. Short-term (<30 min) effects of localized nitric oxide (NO) formation. In rat peritoneal mast cells activated with antigen (Ag) there is increased nuclear and cytoplasmic NO formation. Nuclear NO regulates mRNA expression by moderating transcription factors and controls leukotriene production by nitrosylating 5-lipoxygenase. Cytoplasmic NO interacts with targets like Rac, thus inhibiting degranulation, or cytoskeletal components such as calpain, inhibiting adhesion.

Interestingly, many cGMP-independent effects in MC involve an extended time frame for activity (often up to 18-24 h)(51). Examples from studies in other cells have also shown that the upregulation of iNOS that results in nitrosylation of target proteins requires such a time-course. However, our evidence of short-term (<20 min) NO-mediated inhibition of degranulation and leukotriene release suggests cNOS-mediated mechanisms that involve rapid nitrosylation of target proteins. Identification of proteins that become S-nitrosylated, or that regulate this activity are important avenues of future study (52).

The hypothesis put forward previously concerning the co-expression of two NOS isoforms takes on more significance after localization is taken into account. As eNOS is predominantly a nuclear associated protein in rodent and human MC and iNOS or nNOS are largely cytoplasmic, MC may maintain a NOS protein in the cytoplasm through this co-expession pattern. This mechanism may be particularly relevant after activation, when cytoplasmic eNOS translocates to the nucleus, whereas nNOS or iNOS remains in the cytoplasm. Thus upon activation eNOS may play roles in regulating leukotriene formation or gene expression, while the iNOS or nNOS retained in the cytoplasm may have other roles.

V. Is short-term NO a generator of functional and phenotypic diversity in MC ?:

Microbes have evolved multiple mechanisms for evade immune responses, including high mutation rates and expression of heat-shock and decoy proteins (53). Macrophages and neutrophils, upon activation, elicit diversified innate immune responses against these invading organisms (54,55). Of the many retort mechanisms, production of O_2^- and NO are of vital importance (54). For example, neutrophil O_2^- and NO are involved in clearing Borrelia *sp,* a response that is subdued in neutrophils from NOS deficient mice (54).

As noted above and in Chapter III, there is a short-term NO response in Ag stimulated PMC that likely has several functions. Previously it was shown that PMC "spontaneously" produce NO that contributes to MC cytotoxicity in cooperation with TNF (56). Thus, one of the potential functions may be to provide a population of NO producing MC that respond, not by wholesale degranulation, but

as an accompaniment to TNF release in initiating an innate immune response (Fig. 7.4). Interestingly, LPS-treatment, but not peptidoglycan (PGN) induced a significant percent of PMC to produce NO (>60%)(M. Gilchrist, unpublished). Treatment of mouse and human MC with PGN, but not LPS induced granule mediator release (57,58). LPS may induce a NO response in these cells that mutes degranulation, potentially in favor of releasing selected mediators. NO may thus be operating to generate highly selective mediator release in activated MC populations.

The MC is well known for its release of mediators in metered fashion. TLR activation of mouse MC results in release of different mediators depending if LPS (TNF, IL-1β, IL-6 and IL-13) or PGN (granule mediators, TNF, IL-4, IL-5) is the stimulus (57). In other systems, the plasticity of the MC response is not only stimulus specific, but related to the level of stimulus, with weak antigenic stimuli enhancing a differing spectrum of chemokine production in the absence of degranulation than induced by a strong antigenic stimulus (59). Short-term NO production in PMC may also contribute to this phenomenon (Fig. 7.4). Indeed, MCP-1 production by rat MC was shown to be differentially regulated and explained by a tenet called "kinetic proofreading", which suggests that different ligands induce different effects through binding to the same receptor. Furthermore, the authors proposed a "branch point" in the signaling cascade that generates a soluble, short-lived mediator that contributes to MCP-1 formation and release (60). As the actual contribution of individual MC to chemokine and cytokine release are unknown, the hypothesis can be put forward that these initially NO^{hi} PMC, while being inhibited from degranulating and releasing leukotrienes, may produce and release chemokines or cytokines when NO synthesis abates (Fig. 7.4). A role for nuclear localization of NO that controls gene transcription further supports this postulate. This hypothesis may be further tested by isolation of DAF^{+} and DAF^{-} MC and screening for mediator release in these separated populations.

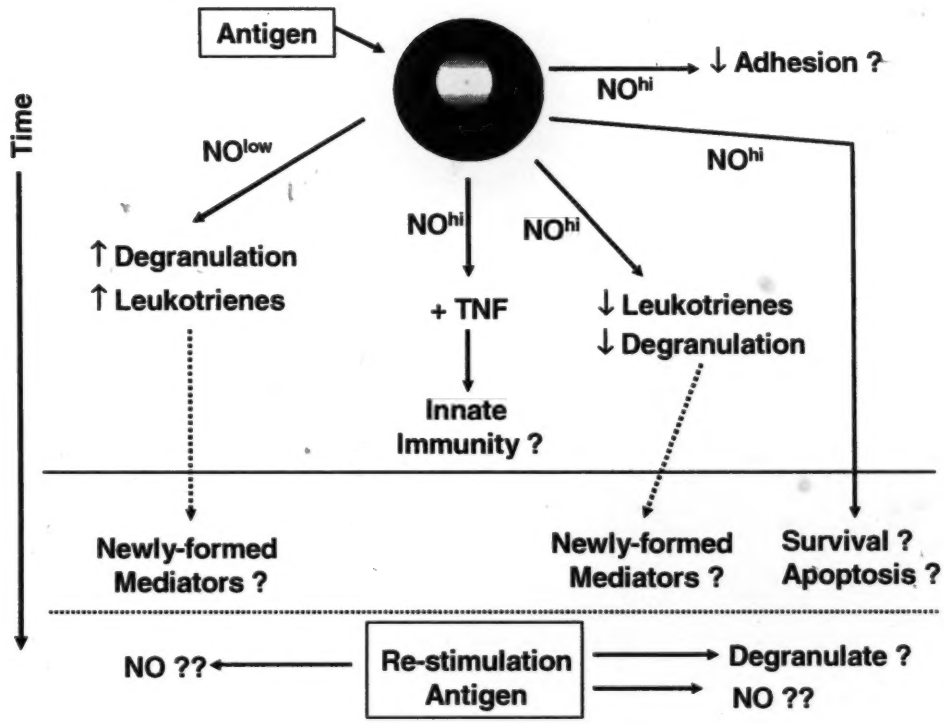


Fig. 7.4. Generation of diversity by short-term nitric oxide (NO) production. Antigen (Ag) stimulation of rat peritoneal mast cells results in a NO^{low} and NO^{hi} populations. The NO^{low} cells degranulate and release leukotrienes. Their cytokine/chemokine release profile is unknown and they may not produce NO if re-stimulated with Ag. NO^{hi} cells can have several potential outcomes. In combination with TNF release, NO may be involved in innate immunity. NO^{hi} PMC may be less responsive due to decreased adhesion or have changes regulating survival or apoptosis. NO^{hi} PMC may be the contributing source of newly-formed mediators such as cytokines. What is not known is if these NO^{hi} cells will degranulate on a later exposure to Ag.

Another potential role for short-term NO formation may be in modulating apoptosis (61). Endothelial cells exposed to shear stress increased NO release by eNOS activity (62) and there was a concordant increase in nitrosylation of cellular proteins including caspase-3 (63). This action inhibited apoptosis in endothelial cells. However in other cell systems NO has a proapoptotic role (Fig. 7.4). These results emphasize the complex role NO plays in cellular mechanisms, and studies directed at the survival and responsiveness of these DAF^+ cells are required.

One important question that was not addressed in these studies was whether the NO^{hi} PMC could be induced to degranulate by Ag stimulation at later time-points. Indeed, as DAF is not ratiometric like many Ca^{2+}-sensing dyes, we were also unable to determine the precise kinetics of NO formation, since a decrease in NO formation in an already DAF positive cell could not be detected (64). Despite these inherent drawbacks, these approaches may help reveal an expanding role for NO in functional plasticity of MC (Fig. 7.4).

VI. Does BH_4 production determine radical species production from MC NOS ?

NOS has obligate requirements for arginine and BH_4 for full activity. Modulation of both of these factors has been shown not only to control NO formation, but also the form of reactive species produced by NOS. Studies using purified NOS proteins suggest that the absence of BH_4 allows O_2 to be the terminal electron acceptor producing O_2^- *in-vitro* (65). *In-vivo* studies in hypertensive rats and BH_4 deficient mouse models showed BH_4 levels directly mediated the ratio of NO to O_2^- produced, and the biological effects of NOS uncoupled by lack of BH_4 were inhibited by catalase and superoxide dismutase further confirming the production of O_2^- (66,67). Recent studies employing direct gene transfer of CHI into either hypertensive or diabetic mice resulted in BH_4-dependent decreased O_2^- production and normalized endothelial function (68,69). These results highlight a important interaction between BH_4 and NOS that directly correlated with control of NO and O_2^- synthesis.

Chapter III describes the critical role for BH_4 in modulating both short-term (<5 min) and extended (>18 h) NO production in MC. Incubation of rat PMC with

IFN-γ resulted in increased iNOS expression, NO production and activity of CHI/BH_4 pathways (Chapter II and III). Pharmacological inhibition of CHI activity almost completely inhibited NO production, an effect that was reconstituted with exogenous BH_4. This diminished NO synthesis was associated with increased responsiveness (β-hex release) in a fashion similar to that shown using NOS inhibitors. Previously we have also shown that stimulation of PMC with anti-CD8 antibody upregulated iNOS production, though NO formation was significantly less than that seen with IFN-γ (6). Anti-CD8 stimulated PMC did not upregulate CHI expression and function, resulting in limited effects on mediator release (M. Gilchrist, unpublished). This data highlighted the significance of BH_4 in fine-tuning MC NO production by both cNOS and iNOS, and implicate CHI and BH_4 as post-translational modifiers of iNOS activity.

Past studies showed that MC are a potential source of reactive oxygen species (ROS) such as O_2^- and H_2O_2 (70,71). These ROS influenced MC function in an opposite manner to NO by sensitizing MC for mediator release, or directly inducing degranulation (72,73). Thus, a balance of O_2^-/NO has been proposed to play a role in modulating MC function (74). Mitochondrial respiration, xanthine oxidase, cycloxygenase, NADPH oxidase or members of the "NOX-1" (non-phagocytic) NADPH oxidases produce ROS (75). Some limited study has identified NADPH oxidase subcomponents in MC, but the critical gp91 oxidase or NOX subunits associated with cellular O_2^- have not been identified (76). Thus, the precise enzymatic origin of ROS in MC remains unresolved.

Incompletely saturated NOS is capable of producing O_2^- and $ONOO^-$, and compelling, yet circumstantial evidence for production of O_2^- via NOS in MC exists in the literature. Treatment of MC with the chemotherapeutic drug, adriamycin, induces histamine release associated with increased O_2^- formation (77). Adriamycin also enhances O_2^- formation from purified eNOS (78).

Treatment of rat PMC with CHI inhibitors also effected short-term (<5 min) NO formation as determined using live-cell confocal analysis (Chapter III). This decreased BH_4 availability resulted not only in depressed NO production, but also hyperresponsiveness, both in increased levels of spontaneous as well as stimulated

mediator (β-hex) release. While requiring further study, it is thus interesting to speculate that inhibition of BH_4 production not only depresses NO formation, but also may enhance O_2^- production that increases MC responsiveness to antigenic stimuli. Such regulation may be a physiological mechanism utilized by MC to aid in degranulation. Further circumstantial evidence from the literature implicates this exact process, as the NOS inhibitor N^{ω}-methyl-L-arginine (L-NMA), while blocking NO formation, maintains the NADPH oxidase (O_2^- producing) activity of the NOS dimer (79). Treatment of rat PMC with this inhibitor increased O_2^- formation with associated potentiation of histamine release (72). Thus BH_4 regulation of NOS may occupy a bifurcation point in controlling free radical production and their associated regulatory outcomes in MC (Fig. 7.5).

The compartmentalization of NOS appears critical to the target specificity of NO and may be further enhanced by the localization of CHI. Since BH_4 is highly labile in biological systems, it has been proposed but not directly shown in endothelial cells that NOS and CHI must also be in close proximity for NO to be produced (69). This concept is analogous to the proposed "metabolon" defined for enzymes responsible for AA metabolism (80). Such enzymes are thought to closely colocalize at plasma membrane and nuclear membrane sites to maximize leukotriene synthesis.

In untreated PMC, CHI staining revealed a previously undescribed membranous localization of CHI with little cytoplasmic staining (Chapter III). Such a staining pattern may be associated with lipid raft interactions. CHI has no consensus sequences for lipid modification, but this does not rule out the possibility of other binding proteins having a role in membrane localization of CHI. Colocalization studies in rat MC lines (RBL-2H3 and RCMC) indicate a strong association between CHI and eNOS proteins, however we have been unable to coprecipitate the two proteins (M. Gilchrist unpublished). Furthermore, IFN-γ treatment increased the cytoplasmic accumulation of CHI analogous to the pattern of iNOS expression

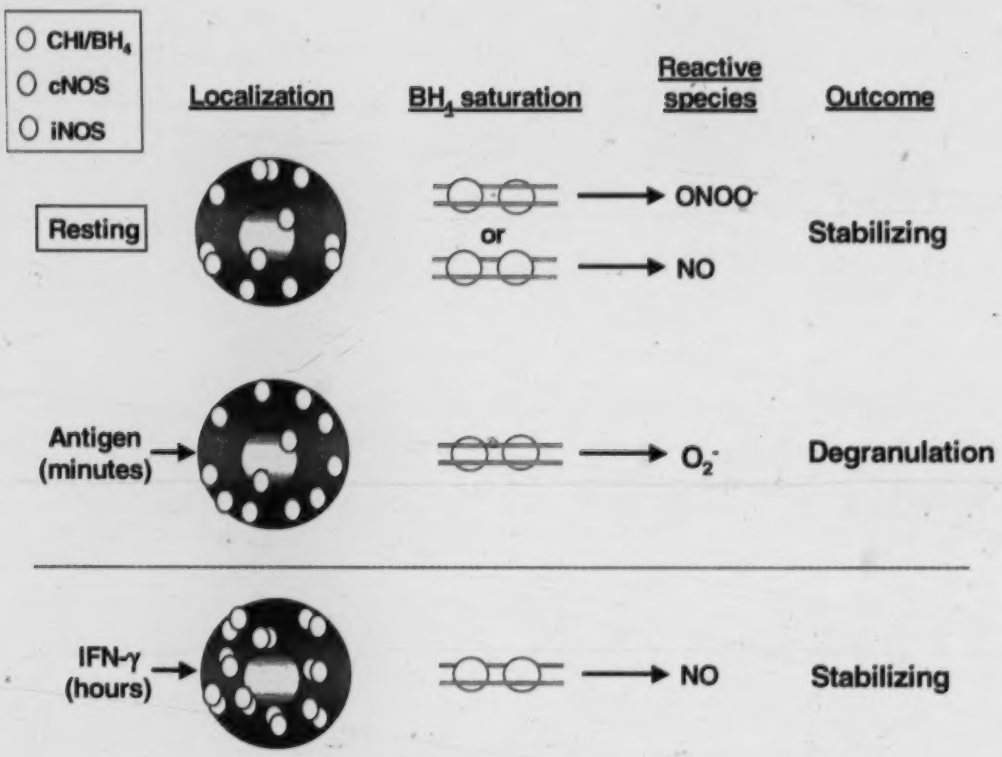


Fig. 7.5. Proposed role for nitric oxide synthase (NOS), GTP-cyclohydrolase I (CHI) and tetrahydrobiopterin (BH_4) in mast cell (MC) activation. The NOS dimer (parallel solid lines) has binding sights for two molecules of BH_4 (open cirlces). Under normal cellular concentrations of BH_4, only one molecule binds constitutive NOS (cNOS), causing the formation of superoxide (O_2^-) as well as nitric oxide (NO), which can combine to produce peroxynitrite ($ONOO^-$), and stabilize the MC membrane. Upon activation, cellular CHI may dissociate from NOS, decreasing local BH_4 concentrations and causing the NOS dimer to produce only O_2^-. O_2^- can potentiate MC degranulation. Several hours after stimulation, or after interferon-γ treatment, MC upregulate CHI levels, resulting in a concordant increase in cellular BH_4. This saturates cellular inducible NOS (iNOS), increasing NO production, which inhibits MC degranulation.

seen in other MC studies (4,6) and similar to the intracellular location of DAF (NO) (Chapter III).

The above observations and the emerging role of NOS formation of O_2^- in host integrity and disease lead to the proposal of a "One-Enzyme" system for NO/O_2^- production in MC based on the dynamic regulation of NOS by BH_4 availability (Fig. 7.5). In this system, cNOS in an unstimulated MC is either partially or fully saturated with BH_4 depending on CHI synthesis and colocalization. In this state cNOS may produce $ONOO^-$ (partial BH4 saturation) or NO (full BH_4 saturation). Upon activation, we propose that signaling molecules are mobilized to lipid rafts which removes CHI from close proximity to cNOS. BH_4 saturation of cNOS is lowered, and O_2^- is the predominant radical now produced, which contributes to MC degranulation. At later time points or after IFN-γ treatment, cellular BH_4 levels are re-established by re-association of NOS and CHI, culminating in increased NO production that inhibits MC degranulation (Fig.7.5). Clearly this hypothesis requires further investigation, including a more detailed study of the cellular distribution of CHI and NOS during activation. Utilization of the hph-1 mouse, which has a deficit in BH_4 production, may aid these studies (81). Taken together these results indicate that CHI may play a critical role in both early degranulation events in MC, as well as long term regulatory events of MC function by NO. Dysregulation of BH_4 levels, or mutations in the BH_4 binding site in NOS may contribute to MC hyperresponsiveness in disease states such as asthma.

VII. Conclusions

Previously, NO has been shown to be a potent regulator of MC secretory function particularly when high exogenous levels are present in the microenvironment (51). Data in this thesis point to the expression of all isoforms of NOS in MC and show that endogenous production of NO inhibits degranulation, leukotriene synthesis and the release of newly synthesized chemokines.

The potential outcomes of endogenous NO production by MC go beyond mediator release and could include roles in MC development, heterogeneity, and functional diversity by specialized sculpting of effector responses. Furthermore, variations in NOS compartmentalization coupled with the identification of dynamic

changes in localized NO production offer novel insights into mechanisms that regulate the amounts, timing and location of its production that can be correlated with potential outcomes. Indeed, identifying nitrosylated targets of NOS as well as the regulatory pathways involved will be of special interest.

Given the broad-spectrum potential of NO, it is now valuable to postulate that MC-derived NO may be involved in all MC-related functions. Certainly NO has been implicated in wound healing, angiogenesis and the regulation of epithelial integrity; MC NO has already been implicated in the latter (82). There is thus, substantial overlap in NO and MC functions and further investigation using knock-out or transfection systems to control the expression or localization of NOS and NO will help re-evaluate the extent of involvement of MC NO in homeostasis. In addition, dysregulation of these important pathways can clearly be a contributing, but as yet unstudied, factor in MC-mediated pathology.

Despite intensive search no fully specific drug have been discovered that inhibits activation of MC (83). Thus, the identification in this thesis of endogenous NO as a regulator of three major facets of MC activation (degranulation, leukotrienes and cytokines) offers a new approach to define pathways that inhibit MC and may hold innovative avenues for pharmaceutical intervention.

References

1. Bidri, M., Feger, F., Varadaradjalou, S., Ben Hamouda, N., Guillosson, J.J., and Arock, M. 2001. Mast cells as a source and target for nitric oxide. *Int. Immunopharmacol.* 1:1543-1558.

2. Eastmond, N.C., Banks, E.M., and Coleman, J.W. 1997. Nitric oxide inhibits IgE-mediated degranulation of mast cells and is the principal intermediate in IFN-gamma-induced suppression of exocytosis. *J. Immunol.* 159:1444-1450.

3. Skaper, S.D., Facci, L., Romanello, S., and Leon, A. 1996. Mast cell activation causes delayed neurodegeneration in mixed hippocampal cultures via the nitric oxide pathway. *J. Neurochem.* 66:1157-1166.

4. Messina, A., Knight, K.R., Dowsing, B.J., Zhang, B., Phan, L.H., Hurley, J.V., Morrison, W.A., and Stewart, A.G. 2000. Localization of inducible nitric oxide synthase to mast cells during ischemia/reperfusion injury of skeletal muscle. *Lab. Invest.* 80:423-431.

5. Shimizu, Y., Sakai, M., Umemura, Y., and Ueda, H. 1997. Immunohistochemical localization of nitric oxide synthase in normal human skin: expression of endothelial-type and inducible-type nitric oxide synthase in keratinocytes. *J. Dermatol.* 24:80-87.

6. Gilchrist, M., Savoie, M., Nohara, O., Wills, F.L., Wallace, J.L., and Befus, A.D. 2002. Nitric oxide synthase and nitric oxide production in in vivo-derived mast cells. *J. Leukoc. Biol.* 71:618-624.

7. Kashiwagi, S., Kajimura, M., Yoshimura, Y., and Suematsu, M. 2002. Nonendothelial source of nitric oxide in arterioles but not in venules: alternative source revealed in vivo by diaminofluorescein microfluorography. *Circ. Res.* 91:e55-e64.

8. Bidri, M., Ktorza, S., Vouldoukis, I., Le Goff, L., Debre, P., Guillosson, J.J., and Arock, M. 1997. Nitric oxide pathway is induced by Fc epsilon RI and up-regulated by stem cell factor in mouse mast cells. *Eur. J. Immunol.* 27:2907-2913.

9. Bacci, S., Arbi-Riccardi, R., Mayer, B., Rumio, C., and Borghi-Cirri, M.B. 1994. Localization of nitric oxide synthase immunoreactivity in mast cells of human nasal mucosa. *Histochemistry* 102:89-92.

10. Wehrle-Haller, B. 2003. The role of kit-ligand in melanocyte development and epidermal homeostasis. *Pigment Cell. Res.* 16:287-296.

11. Ahmed, B. and Van Den Oord, J.J. 1999. Expression of the neuronal isoform of nitric oxide synthase (nNOS) and its inhibitor, protein inhibitor of nNOS, in pigment cell lesions of the skin. *Br. J. Dermatol.* 141:12-19.

12. Altdorfer, K., Bagameri, G., Donath, T., and Feher, E. 2002. Nitric oxide synthase immunoreactivity of interstitial cells of Cajal in experimental colitis. *Inflamm. Res.* 51:569-71.

13. Maruo, K., Kayashima, K.I., and Ono, T. 1999. Expression of neuronal nitric oxide synthase in dermal infiltrated eosinophils in eosinophilic pustular folliculitis. *Br. J. Dermatol.* 140:417-420.

14. Kirshenbaum, A.S., Akin, C., Wu, Y., Rottem, M., Goff, J.P., Beaven, M.A., Rao, V.K., and Metcalfe, D.D. 2003. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. *Leuk. Res.* 27:677-682.

15. Weinberg, J.B., Misukonis, M.A., Shami, P.J., Mason, S.N., Sauls, D.L., Dittman, W.A., Wood, E.R., Smith, G.K., McDonald, B., and Bachus, K.E. 1995. Human mononuclear phagocyte inducible nitric oxide synthase (iNOS):

analysis of iNOS mRNA, iNOS protein, biopterin, and nitric oxide production by blood monocytes and peritoneal macrophages. *Blood* 86:1184-1195.

16. Taylor, B.S., Alarcon, L.H., and Billiar, T.R. 1998. Inducible nitric oxide synthase in the liver: regulation and function. *Biochemistry (Mosc.)* 63:766-781.

17. Feng, X., Guo, Z., Nourbakhsh, M., Hauser, H., Ganster, R., Shao, L., and Geller, D.A. 2002. Identification of a negative response element in the human inducible nitric-oxide synthase (hiNOS) promoter: The role of NF-kappa B-repressing factor (NRF) in basal repression of the hiNOS gene. *Proc. Natl. Acad. Sci. U. S. A* 99:14212-14217.

18. Fang, F.C. and Vazquez-Torres, A. 2002. Nitric oxide production by human macrophages: there's NO doubt about it. *Am. J. Physiol. Lung Cell Mol. Physiol.* 282:L941-L943.

19. Koda, W., Harada, K., Tsuneyama, K., Kono, N., Sasaki, M., Matsui, O., and Nakanuma, Y. 2000. Evidence of the participation of peribiliary mast cells in regulation of the peribiliary vascular plexus along the intrahepatic biliary tree. *Lab. Invest.* 80:1007-1017.

20. Andrew, P.J. and Mayer, B. 1999. Enzymatic function of nitric oxide synthases. *Cardiovasc. Res.* 43:521-531.

21. Connelly, L., Jacobs, A.T., Palacios-Callender, M., Moncada, S., and Hobbs, A.J. 2003. Macrophage endothelial nitric-oxide synthase autoregulates cellular activation and pro-inflammatory protein expression. *J. Biol. Chem.* 278:26480-26487.

22. De Sanctis, G.T., MacLean, J.A., Hamada, K., Mehta, S., Scott, J.A., Jiao, A., Yandava, C.N., Kobzik, L., Wolyniec, W.W., Fabian, A.J., Venugopal, C.S., Grasemann, H., Huang, P.L., and Drazen, J.M. 1999. Contribution of nitric

oxide synthases 1, 2, and 3 to airway hyperresponsiveness and inflammation in a murine model of asthma. *J. Exp. Med.* 189:1621-1630.

23. Masini, E., Salvemini, D., Pistelli, A., Mannaioni, P.F., and Vane, J.R. 1991. Rat mast cells synthesize a nitric oxide like-factor which modulates the release of histamine. *Agents Actions* 33:61-63.

24. Hogaboam, C.M., Befus, A.D., and Wallace, J.L. 1993. Modulation of rat mast cell reactivity by IL-1 beta. Divergent effects on nitric oxide and platelet-activating factor release. *J. Immunol.* 151:3767-3774.

25. Koranteng, R.D., Dearman, R.J., Kimber, I., and Coleman, J.W. 2000. Phenotypic variation in mast cell responsiveness to the inhibitory action of nitric oxide. *Inflamm. Res.* 49:240-246.

26. Michell, B.J., Chen, Z., Tiganis, T., Stapleton, D., Katsis, F., Power, D.A., Sim, A.T., and Kemp, B.E. 2001. Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. *J. Biol. Chem.* 276:17625-17628.

27. Fleming, I. and Busse, R. 2003. Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 284:R1-12.

28. Suzuki, N., Kojima, H., Urano, Y., Kikuchi, K., Hirata, Y., and Nagano, T. 2002. Orthogonality of calcium concentration and ability of 4,5-diaminofluorescein to detect NO. *J. Biol. Chem.* 277:47-49.

29. Smukler, S.R., Tang, L., Wheeler, M.B., and Salapatek, A.M. 2002. Exogenous nitric oxide and endogenous glucose-stimulated beta-cell nitric oxide augment insulin release. *Diabetes* 51:3450-3460.

30. Zhang, D.X., Yi, F.X., Zou, A.P., and Li, P.L. 2002. Role of ceramide in TNF-alpha-induced impairment of endothelium- dependent vasorelaxation in coronary arteries. *Am. J. Physiol. Heart Circ. Physiol.* 283:H1785-94

31. Qiu, H., Orr, F.W., Jensen, D., Wang, H.H., McIntosh, A.R., Hasinoff, B.B., Nance, D.M., Pylypas, S., Qi, K., Song, C., Muschel, R.J., and Al Mehdi, A.B. 2003. Arrest of B16 melanoma cells in the mouse pulmonary microcirculation induces endothelial nitric oxide synthase-dependent nitric oxide release that is cytotoxic to the tumor cells. *Am. J. Pathol.* 162:403-412.

32. Grisham, M.B., Jourd'Heuil, D., and Wink, D.A. 1999. Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites:implications in inflammation. *Am. J. Physiol.* 276:G315-G321.

33. Fulton, D., Gratton, J.P., and Sessa, W.C. 2001. Post-translational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough? *J. Pharmacol..Exp. Ther.* 299:818-824.

34. Webb, J.L., Harvey, M.W., Holden, D.W., and Evans, T.J. 2001. Macrophage nitric oxide synthase associates with cortical actin but is not recruited to phagosomes. *Infect. Immun.* 69:6391-6400.

35. Bandaletova, T., Brouet, I., Bartsch, H., Sugimura, T., Esumi, H., and Ohshima, H. 1993. Immunohistochemical localization of an inducible form of nitric oxide synthase in various organs of rats treated with Propionibacterium acnes and lipopolysaccharide. *APMIS* 101:330-336.

36. Barker, J.E., Knight, K.R., Romeo, R., Hurley, J.V., Morrison, W.A., and Stewart, A.G. 2001. Targeted disruption of the nitric oxide synthase 2 gene protects against ischaemia/reperfusion injury to skeletal muscle. *J. Pathol.* 194:109-115.

37. Wills, F.L., Gilchrist, M., and Befus, A.D. 1999. Interferon-gamma regulates the interaction of RBL-2H3 cells with fibronectin through production of nitric oxide. *Immunology* 97:481-489.

38. Forsythe, P. and Befus, A.D. 2003. Inhibition of calpain is a component of nitric oxide-induced down- regulation of human mast cell adhesion. *J. Immunol.* 170:287-293.

39. Rohn, T.T., Nelson, L.K., Davis, A.R., and Quinn, M.T. 1999. Inhibition of GTP binding to Rac2 by peroxynitrite: potential role for tyrosine modification. *Free Radic. Biol. Med.* 26:1321-1331.

40. Bissonnette, E.Y., Chin, B., and Befus, A.D. 1995. Interferons differentially regulate histamine and TNF-alpha in rat intestinal mucosal mast cells. *Immunology* 86:12-17.

41. Blottner, D. and Luck, G. 2001. Just in time and place: NOS/NO system assembly in neuromuscular junction formation. *Microsc. Res. Tech.* 55:171-180.

42. Prabhakar, P., Cheng, V., and Michel, T. 2000. A chimeric transmembrane domain directs endothelial nitric-oxide synthase palmitoylation and targeting to plasmalemmal caveolae. *J. Biol. Chem.* 275:19416-19421.

43. Giordano, A., Tonello, C., Bulbarelli, A., Cozzi, V., Cinti, S., Carruba, M.O., and Nisoli, E. 2002. Evidence for a functional nitric oxide synthase system in brown adipocyte nucleus. *FEBS Lett.* 514:135-140.

44. Buchwalow, I.B., Schulze, W., Karczewski, P., Kostic, M.M., Wallukat, G., Morwinski, R., Krause, E.G., Muller, J., Paul, M., Slezak, J., Luft, F.C., and Haller, H. 2001. Inducible nitric oxide synthase in the myocard. *Mol. Cell. Biochem.* 217:73-82.

45. Govers, R., van der, S.P., van Donselaar, E., Slot, J.W., and Rabelink, T.J. 2002. Endothelial nitric oxide synthase and its negative regulator caveolin-1 localize to distinct perinuclear organelles. *J. Histochem. Cytochem.* 50:779-788.

46. Brock, T.G., McNish, R.W., and Peters-Golden, M. 1995. Translocation and leukotriene synthetic capacity of nuclear 5-lipoxygenase in rat basophilic leukemia cells and alveolar macrophages. *J. Biol. Chem.* 270:21652-21658.

47. Coffey, M.J., Phare, S.M., and Peters-Golden, M. 2002. Interaction between nitric oxide, reactive oxygen intermediates, and peroxynitrite in the regulation of 5-lipoxygenase metabolism. *Biochim. Biophys. Acta.* 1584:81-90.

48. Shin, J.S., Gao, Z., and Abraham, S.N. 2000. Involvement of cellular caveolae in bacterial entry into mast cells. *Science* 289:785-788.

49. Fulton, D., Fontana, J., Sowa, G., Gratton, J.P., Lin, M., Li, K.X., Michell, B., Kemp, B.E., Rodman, D., and Sessa, W.C. 2002. Localization of endothelial nitric-oxide synthase phosphorylated on serine 1179 and nitric oxide in Golgi and plasma membrane defines the existence of two pools of active enzyme. *J. Biol. Chem.* 277:4277-4284.

50. Stauffer, T.P. and Meyer, T. 1997. Compartmentalized IgE receptor-mediated signal transduction in living cells. *J. Cell. Biol.* 139:1447-1454.

51. Coleman, J.W. 2002. Nitric oxide: a regulator of mast cell activation and mast cell- mediated inflammation. *Clin. Exp. Immunol.* 129:4-10.

52. Matsumoto, A., Comatas, K.E., Liu, L., and Stamler, J.S. 2003. Screening for nitric oxide-dependent protein-protein interactions. *Science* 301:657-661.

53. Vazquez-Torres, A., Jones-Carson, J., Mastroeni, P., Ischiropoulos, H., and Fang, F.C. 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. *J. Exp. Med.* 192:227-236.

54. Lusitani, D., Malawista, S.E., and Montgomery, R.R. 2002. Borrelia burgdorferi are susceptible to killing by a variety of human polymorphonuclear leukocyte components. *J. Infect. Dis.* 185:797-804.

55. Alam, M.S., Akaike, T., Okamoto, S., Kubota, T., Yoshitake, J., Sawa, T., Miyamoto, Y., Tamura, F., and Maeda, H. 2002. Role of nitric oxide in host defense in murine salmonellosis as a function of its antibacterial and antiapoptotic activities. *Infect. Immun.* 70:3130-3142.

56. Bissonnette, E.Y., Hogaboam, C.M., Wallace, J.L., and Befus, A.D. 1991. Potentiation of tumor necrosis factor-alpha-mediated cytotoxicity of mast cells by their production of nitric oxide. *J. Immunol.* 147:3060-3065.

57. Supajatura, V., Ushio, H., Nakao, A., Akira, S., Okumura, K., Ra, C., and Ogawa, H. 2002. Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity. *J. Clin. Invest.* 109:1351-1359.

58. Suzuki, K., Hirose, T., Matsuda, H., Hasegawa, S., Okumura, K., and Ra, C. 1998. The Fc receptor (FcR) gamma subunit is essential for IgE-binding activity of cell-surface expressed chimeric receptor molecules constructed from human high-affinity IgE receptor (Fc epsilon RI) alpha and FcR gamma subunits. *Mol. Immunol.* 35:259-270.

59. Gonzalez-Espinosa, C., Odom, S., Olivera, A., Hobson, J.P., Martinez, M.E., Oliveira-Dos-Santos, A., Barra, L., Spiegel, S., Penninger, J.M., and Rivera, J. 2003. Preferential signaling and induction of allergy-promoting lymphokines upon weak stimulation of the high affinity IgE receptor on mast cells. *J. Exp. Med.* 197:1453-1465.

60. Eglite, S., Morin, J.M., and Metzger, H. 2003. Synthesis and secretion of monocyte chemotactic protein-1 stimulated by the high affinity receptor for IgE. *J. Immunol.* 170:2680-2687.

61. Forsythe, P., Gilchrist, M., Kulka, M., and Befus, A.D. 2001. Mast cells and nitric oxide: control of production, mechanisms of response. *Int. Immunopharmacol.* 1:1525-1541.

62. Hoffmann, J., Dimmeler, S., and Haendeler, J. 2003. Shear stress increases the amount of S-nitrosylated molecules in endothelial cells: important role for signal transduction. *FEBS Lett.* 551:153-158.

63. Mannick, J.B. and Schonhoff, C.M. 2002. Nitrosylation: the next phosphorylation? *Arch. Biochem. Biophys.* 408:1-6.

64. Yi, F.X., Zhang, A.Y., Campbell, W.B., Zou, A.P., Van Breemen, C., and Li, P.L. 2002. Simultaneous in situ monitoring of intracellular Ca2+ and NO in endothelium of coronary arteries. *Am. J. Physiol. Heart Circ. Physiol.* 283:H2725-H2732.

65. Rosen, G.M., Tsai, P., Weaver, J., Porasuphatana, S., Roman, L.J., Starkov, A.A., Fiskum, G., and Pou, S. 2002. The role of tetrahydrobiopterin in the regulation of neuronal nitric-oxide synthase-generated superoxide. *J. Biol. Chem.* 277:40275-40280.

66. Cosentino, F., Patton, S., d'Uscio, L.V., Werner, E.R., Werner-Felmayer, G., Moreau, P., Malinski, T., and Luscher, T.F. 1998. Tetrahydrobiopterin alters superoxide and nitric oxide release in prehypertensive rats. *J. Clin. Invest.* 101:1530-1537.

67. Cosentino, F., Barker, J.E., Brand, M.P., Heales, S.J., Werner, E.R., Tippins, J.R., West, N., Channon, K.M., Volpe, M., and Luscher, T.F. 2001. Reactive oxygen species mediate endothelium-dependent relaxations in tetrahydrobiopterin-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 21:496-502.

68. Alp, N.J., Mussa, S., Khoo, J., Cai, S., Guzik, T., Jefferson, A., Goh, N., Rockett, K.A., and Channon, K.M. 2003. Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase I overexpression. *J. Clin. Invest.* 112:725-735.

69. Zheng, J.S., Yang, X.Q., Lookingland, K.J., Fink, G.D., Hesslinger, C., Kapatos, G., Kovesdi, I., and Chen, A.F. 2003. Gene transfer of human guanosine 5'-triphosphate cyclohydrolase I restores vascular tetrahydrobiopterin level and endothelial function in low renin hypertension. *Circulation* 108:1238-1245.

70. Yoshimaru, T., Suzuki, Y., Matsui, T., Yamashita, K., Ochiai, T., Yamaki, M., and Shimizu, K. 2002. Blockade of superoxide generation prevents high-affinity immunoglobulin E receptor-mediated release of allergic mediators by rat mast cell line and human basophils. *Clin. Exp. Allergy* 32:612-618.

71. Guerin-Marchand, C., Senechal, H., Pelletier, C., Fohrer, H., Olivier, R., David, B., Berthon, B., and Blank, U. 2001. H2O2 impairs inflammatory mediator release from immunologically stimulated RBL-2H3 cells through a redox-sensitive, calcium-dependent mechanism. *Inflamm. Res.* 50:341-349.

72. Brooks, A.C., Whelan, C.J., and Purcell, W.M. 1999. Reactive oxygen species generation and histamine release by activated mast cells: modulation by nitric oxide synthase inhibition. *Br. J. Pharmacol.* 128:585-590.

73. Peden, D.B., Dailey, L., DeGraff, W., Mitchell, J.B., Lee, J.G., Kaliner, M.A., and Hohman, R.J. 1994. Hydrogen peroxide effects on rat mast cell function. *Am. J. Physiol.* 267:L85-L93.

74. Niu, X.F., Ibbotson, G., and Kubes, P. 1996. A balance between nitric oxide and oxidants regulates mast cell- dependent neutrophil-endothelial cell interactions. *Circ. Res.* 79:992-999.

75. Griendling, K.K., Sorescu, D., and Ushio-Fukai, M. 2000. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ. Res.* 86:494-501.

76. Fukuishi, N., Sakaguchi, M., Matsuura, S., Nakagawa, C., Akagi, R., and Akagi, M. 1997. The mechanisms of compound 48/80-induced superoxide

generation mediated by A-kinase in rat peritoneal mast cells. *Biochem. Mol. Med.* 61:107-113.

77. Estevez, M.D., Vieytes, M.R., and Botana, L.M. 1994. Study of the activation mechanism of adriamycin on rat mast cells. *Agents Actions* 42:86-91.

78. Vasquez-Vivar, J., Martasek, P., Hogg, N., Masters, B.S., Pritchard, K.A., Jr., and Kalyanaraman, B. 1997. Endothelial nitric oxide synthase-dependent superoxide generation from adriamycin. *Biochemistry* 36:11293-11297.

79. Wang, W., Wang, S., Yan, L., Madara, P., Del Pilar, C.A., Wesley, R.A., and Danner, R.L. 2000. Superoxide production and reactive oxygen species signaling by endothelial nitric-oxide synthase. *J. Biol. Chem.* 275:16899-16903.

80. Peters-Golden, M. and Brock, T.G. 2001. Intracellular compartmentalization of leukotriene synthesis: unexpected nuclear secrets. *FEBS Lett.* 487:323-326.

81. Canevari, L., Land, J.M., Clark, J.B., and Heales, S.J. 1999. Stimulation of the brain NO/cyclic GMP pathway by peripheral administration of tetrahydrobiopterin in the hph-1 mouse. *J. Neurochem.* 73:2563-2568.

82. Kanwar, S., Wallace, J.L., Befus, D., and Kubes, P. 1994. Nitric oxide synthesis inhibition increases epithelial permeability via mast cells. *Am. J. Physiol.* 266:G222-G229.

83. Forsythe, P. and Ennis, M. 2000. Clinical consequences of mast cell heterogeneity. *Inflamm. Res.* 49:147-154.

84. Hunt, J.S., Miller, L., Vassmer, D., and Croy, B.A. 1997. Expression of the inducible nitric oxide synthase gene in mouse uterine leukocytes and potential relationships with uterine function during pregnancy. *Biol. Reprod.* 57:827-836.

85. Kitajima, I., Kawahara, K., Nakajima, T., Soejima, Y., Matsuyama, T., and Maruyama, I. 1994. Nitric oxide-mediated apoptosis in murine mastocytoma. *Biochem. Biophys. Res. Commun.* 204:244-251.